The effect of spatial heterogeneity on nitrate reduction in soil systems

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Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Technical University of Denmark

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PhD Thesis
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Printed by: GraphicCo
August 2015

Cover: Torben Dolin

Preface

This thesis is based on a PhD project carried out at the Department of Environmental Engineering of the Technical University of Denmark from December 2011 to May 2015. The project was conducted under the supervision of Barth F. Smets (DTU Environment), co-supervised by Arnaud Dechesne (DTU Environment) and Rasmus Jakobsen (GEUS).

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I **Pedersen, L. L.**, Dechesne, A., Jensen, M. M., Smets, B. F. (2015) Dissimilatory Nitrate Reduction to Ammonium in *Fagus sylvatica* forest soil. *Manuscript in preparation.*

II **Pedersen, L. L.**, Dechesne, A., Jensen, M. M., Smets, B. F. (2015) Reducing diffusion limitation shifts nitrate reduction metabolism from incomplete denitrification to reduction to ammonium. *Manuscript in preparation.*

III **Pedersen, L. L.**, Smets, B. F., Dechesne, A. (2015) Measuring biogeochemical heterogeneity at the micro scale in soil and sediments. *Manuscript under review.*

IV **Pedersen, L. L.**, Dechesne, A., Smets, B. F. (2015) A nitrate sensitive planar optode; performance & interferences. *Manuscript under review.*

V **Pedersen, L. L.**, Smets, B. F., Dechesne, A. (2015) Notification of Invention at DTU: Planar optode sensor sheet production kit.

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

In addition, the following presentations at international conferences were also concluded during this PhD study:

Pedersen, L. L., Dechesne, A., Smets, B. F. (2014) Reducing diffusion limitation shifts the dominant nitrate reduction metabolism from incomplete denitrification to DNRA. *Oral presentation.* Biogeochemical Interfaces in Soil - Towards a Comprehensive and Mechanistic Understanding of Soil Functions, Leipzig, Germany.

Pedersen, L. L., Dechesne, A., Smets, B. F. (2014) Reducing diffusion limitation shifts the dominant nitrate reduction pathway from incomplete denitrification to DNRA. *Poster presentation.* Danish Microbiological Society Congress 2014, Copenhagen, Denmark.

These presentations are not explicitly considered in this thesis.

Acknowledgements

First of all, I would like to thank senior researcher Rasmus Jakobsen for taking on the mantle of supervisor when the position became vacant a few weeks into my project, and for professor Barth F. Smets to accept the position a year later when Rasmus moved on to work at the Geological Survey of Denmark and Greenland, GEUS. I am grateful to Barth for fostering an intellectually productive environment with his abundant enthusiasm, encouragements and advice. I would also like to thank my co-supervisor, senior researcher Arnaud Dechesne for his always calm disposition and plentiful insightful guidance.

I would like to express my gratitude to Bent Skov, Lene K. Jensen, Hector A. Diaz, Sinh H. Nguyen for their technical and moral support, Torben Dolin for help with the graphical aspects of the thesis, Hugo Connery and Rene Leps for IT support, and Anne Harsting for resolving many administrative issues.

May 31st, 2015, Kongens Lyngby

Summary

Nitrogen is not only an abundant element on earth, making up roughly 80% of the earth's atmosphere, it is also essential for life, and a functional nitrogen cycle is of great importance to human activities and our ecosystems. The nitrogen cycle ultimately returns reactive nitrogen, which was chemically or biochemically fixed from inert nitrogen, back into the atmosphere as inert nitrogen. Over the last century, the excess of anthropogenically fixed nitrogen has put increasing pressures on the nitrogen cycle. Nitrate is a central molecule in the nitrogen cycle. Its concentration is, on the one hand governed by formation by oxidation of ammonia-N, and on the other hand by removal a removal by two dissimilatory nitrate reduction processes: denitrification, in which nitrate is converted to the gaseous compounds dinitrogen and nitrous oxide, and dissimilatory nitrate reduction to ammonium, DNRA. While both processes bring about the reduction of nitrate, their impact on ecosystems is radically different – especially in soil environments. Nitrate itself is poorly retained in soils, and its conversion to gaseous dinitrogen and nitrous oxide through denitrification only serves to further the loss of reactive nitrogen from the system. On top of that nitrous oxide is an important air pollutant and greenhouse gas, with a global warming potential per unit mass 300 times higher than carbon dioxide. DNRA, on the other hand, converts nitrate to ammonium, which is more easily retained in soils than nitrate, and can be assimilated into organic matter, effectively bypassing both denitrification and dinitrogen fixation and conserving nitrogen in the ecosystem.

It is well established that soil is an extremely heterogeneous environment, not merely on a macroscopic level, but also on a microscopic level. Spatial heterogeneity and diffusive limitations result in the formation of specialized niches. It is becoming increasingly clear that these factors are of great importance for biogeochemical processes such as the carbon cycle. Studying the heterogeneity of soil and its impact on ecological processes is not merely a fascinating scientific activity, it may very well be central to gaining insights to influence fundamental soil processes such as nitrogen metabolism, promising advancement of agricultural and pollution prevention and remediation techniques.

A number of conceptual and quantitative frameworks have been developed to assess the impact of mass transfer kinetics on biotransformation rates in various environments. One such approach uses the dimensionless parameter

Da_3: a Damköhler number which for a given system quantifies the relative impact of diffusive limitation on biotransformation.

During this PhD project, we specifically examined the incidence of denitrification and DNRA in soil systems and studied the impact of diffusive limitation on their relative occurrence. An array of column-based soil microcosms was set up to look at the incidence and magnitude of DNRA vs. denitrification in *Fagus sylvatica* forest soil litter, and to investigate the effect of electron donor abundance and bacterial inoculum size on nitrate reduction. Increasing the electron donor abundance increased both DNRA and denitrification, allowing both processes to coexist in the system. At reduced biokinetic limitations, obtained by increasing the initial inoculum size, nitrate reduction was barely affected, but DNRA increased substantially by 71%. Additionally, nitrite-, ammonium-, and nitrous oxide were sequentially produced during nitrate reduction: an initial burst of nitrite production led to DNRA, and for the microcosms which became mass transfer limited also to nitrous oxide production.

To allow application of the Damköhler number framework, a well-controlled experimental system was required where interfacial areas of diffusion are well defined. Hence, a protocol for encasing soil material in alginate aggregates with well-defined size and geometry was developed. The degree of diffusive limitation was then imposed using soil-alginate aggregates with different defined Da_3 values. These were applied in an array of column microcosms to investigate the effect of diffusive limitation on nitrate reduction processes. Going from a high to a low degree of diffusive limitation shifted the system from denitrification, with significant release of nitrous oxide, to DNRA. Carefully imposed degrees of diffusive limitations are powerful tools to studying environmental processes. These results clearly reveal heterogeneity in the form of diffusive limitation can impact nitrate reduction processes. Our results also indicate that a simple management scenario that would allow retention of reactive nitrogen in soil (favouring DNRA over denitrification) would involve adequate soil mixing after addition of excess of electron donor substrate.

In addition to a contribution to the primary literature, an exhaustive review was conducted on tools applicable to collecting geochemical data at the microscale in soil and sediment. The review examined their ability to provide spatially resolved data with microscale resolution, focusing on their performance characteristics, the degree of physical disruption they inflict on

the system being studied, the potential for repeated measurements and the accessibility of the tools. In addition to providing an overview of existing tools, the review revealed that many parts of the microscale toolkit have become increasingly accessible and affordable. However work remains to be done to facilitate simultaneous measurements of multiple analytes, and to expand the array of potential analytes for the various techniques.

In addition, during this PhD study, a nitrate sensitive planar optode was developed along with a tool for producing large, smooth planar optode sensor foil sheets. This is the first planar optode of its kind, and it exhibits a linear response to nitrate from 1 to 50 mM at pH 8.0, a fast response time of < 10s and good lifetime, allowing for fast two dimensional measurements of nitrate distributions over long periods of time. This new sensor technology allows dynamic two dimensional measurements of nitrate at microscale spatial resolution. Unfortunately, time did not permit to use the optode after its development.

Dansk sammenfatning

Nitrogen er ikke alene et meget udbredt element på jorden, udgørende godt og vel 80% af atmosfæren, det er også essentielt for liv, og en funktionel nitrogencyklus er af stor betydning for menneskelige aktiviteter og vore økosystemer. Nitrogencyklussen returnerer reaktivt nitrogen, som tidligere var kemiskt eller biologiskt fikseret fra ureaktivt nitrogen, til atmosfæren i form af ureaktivt nitrogen. I løbet af det sidste århundrede har antropogeniskt fikseret nitrogen sat pres på nitrogencyklussen. Nitrat er et af de centrale molekyler i nitrogencyklussen. Dets koncentration er på den ene hånd styret af dets dannelse via oxidation af ammoniak-N, og på den anden hånd af dets omdannelse som følge af to dissimilatoriske nitratreduktionsprocesser: Denitrifikation, der omdanner nitrat til gasserne dinitrogen og dinitrogenoxid, og dissimilatorisk nitratreduktion til ammonium, DNRA. Mens begge processer medfører reduktion af nitrat, så er deres effekt på økosystemer meget forskellige - især i jordøkosystemer. Nitrat selv bliver kun svagt tilbageholdt i jord, og dets omdannelse til gasserne dinitrogen og dinitrogenoxid via denitrifikation fører til yderligere tab af reaktivt nitrogen fra systemet. Ydermere så bidrager dinitrogenoxid til luftforurening, og er en drivhusgas med et globalt opvarmningspotentiale per masseenhed 300 gange højere en kuldioxid. DNRA, derimod, omdanner nitrat til ammonium, som bliver lettere tilbageholdt i jord end nitrat, og kan blive assimileret ind i organiske forbindelser, hvilket fører til en kortslutning af nitrogencyklussen ved at gå uden om både denitrifikation og fiksering af dinitrogen, hvilket fører til bevaring af nitrogen i økosystemet.

Det er allerede veletableret at jord er et særdeles heterogent miljø, ikke alene makroskopiskt, men også på mikroskopiskt niveau. Rumlig heterogenitet og diffusive begrænsninger fører til dannelse af specialiserede nicher. På nuværende tidspunkt bliver det tiltagende åbenlyst at disse faktorer er af stor betydning for vigtige biogeokemiske processer såsom kulstofcyklussen. Studiet af jords heterogenitet og dets betydning for forskellige processer er ikke alene en fascinerende videnskabelig beskæftigelse, det kan meget vel være centralt for bibringelsen af viden der kan muliggøre at vi kan påvirke grundlæggende processer i jord såsom nitrogenmetabolismen, hvilket lover fremskridt inden for agrikultur og forebyggelse samt oprydning af forurening.

Et antal konceptuelle og kvantitative systemer er blevet udviklet for at kunne bedømme betydningen af massebalance kinetik på biotransformationshastigheder i forskellige miljøer. Et af disse systemer

omfatter den dimensionsløse parameter Da_3, et Damköhler tal som for et givent system kvantificerer den relative effekt af diffusiv begrænsning på biotransformation.

I dette PhD projekt undersøgte vi specifikt forekomsten og omfanget af denitrifikation og DNRA i jordsystemer, og studerede effekten af diffusiv begrænsning på deres relative forekomster. En række søjlesystemer blev sat op for at se forekomsten og omfanget af DNRA vs. denitrifikation i *Fagus sylvatica* skovjord, samt effekten af mængden af tilgængelig elektrondonor og bakterielt inokulum på nitratreduktion. En forøgelse af mængden af elektrondonor øgede både DNRA og denitrifikation, og fik begge processer til forløbe på samme tid i systemet. At gøre systemet mindre biokinetiskt begrænset ved at øge inokulum medførte begrænset forøgelse af nitratreduktionen, samt en betydelig forøgelse af DNRA-aktivitet på 71%. Desuden sås nitrit-, ammonium- og dinitrogenoxidproduktion at følge et sekventielt mønster under nitratreduktion: En indledende svag nitritproduktion blev efterfulgt af DNRA, og for de søjlesystemer som nåede at blive begrænsede af nitrat også dinitrogenoxidproduktion.

Et velkontrolleret eksperimentelt system med veldefinerede grænseflader er påkrævet for at kunne benytte Damköhler systemet. Følgelig blev en protokol for at indbygge jord i alginatpartikler med veldefinerede størrelser og geometrier udviklet. Graden af diffusiv begrænsning blev efterfølgende påført systemer ved hjælp af jord-alginatpartikler med forskellige, veldefinerede Da_3-værdier. Disse blev brugt i en række søjlesystemer til at undersøge effekten af diffusiv begrænsning på nitratreduktionsprocesser. At gå fra en høj grad af diffusiv begrænsning til en lav sås at flytte systemet fra denitrifikation med betydelig frigivelse af dinitrogenoxid til DNRA. Forsigtigt påførte grader af diffusiv begrænsning er et kraftfuldt værktøj i studiet af miljøprocesser. Forsøgets resultater viser klart at heterogenitet i form af diffusiv begrænsning kan påvirke nitratreduktionsprocesser. Vores resultater peger også på at et simpelt management scenario der tillader tilbageholdelse af reaktivt nitrogen i jord (ved at fremme DNRA frem for denitrifikation) vil omfatte tilpas blanding af jord efter tilsættelse af overskud af elektrondonorsubstrat.

Ud over bidraget til primærlitteraturen blev en omfattende gennemgang af værktøj der tillader indhentning af geokemiske data med mikrometeropløsning i jord og sediment udført. Gennemgangen undersøgte værktøjernes ydeevnekarakteristika, i hvilken grad de forstyrrer det

undersøgte system, muligheden for at foretage gentagne målinger samt værktøjernes tilgængelighed. Ud over at give et overblik over tilgængelige værktøj, der viste gennemgangen at mange af dem er blevet tiltagende mere tilgængelige. Dog skal der gøres mere for at gøre det nemmere at måle flere analytter på samme tid, samt for at øge antallet af analytter der kan måles med de enkelte teknikker.

Desuden blev der under PhD studiet udviklet en nitratsensitiv plan optode sammen med et værktøj der muliggør produktionen af store, glatte, plane optode sensorark. Dette er den første planare optode af sin art, den udviser et lineært signal for nitrat fra 1 til 50 mM ved pH 8.0, en kort responstid på < 10s og en god holdbarhed. Denne nye sensorteknologi muliggør dynamiske, todimensionelle målinger af nitratfordelinger med mikrometeropløsning over lange tidsrum. Desværre tillod den tilbageværende tid ikke brug af optoden efter dens udvikling.

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Abbreviations and symbols

Ag	Silver
Anammox	ANaerobic AMMonium OXidation
As	Arsenic
Br	Bromine
Ca	Calcium
$CaCl_2$	Calcium chloride
Cd	Cadmium
Cl	Chlorine
Co	Cobalt
CO_2	Carbon dioxide
Cr	Chromium
Cs^+	Caesium ion
CT	Computer aided x-ray Tomography
Cu	Copper
Da_3	Third Damköhler number
D_{AB}	Diffusion coefficient of *A* in *B*
D_{eff}	Effective intra-grain diffusion coefficient
DET	diffusive equilibrium in thin-film
DGT	diffusive gradient in thin-film
DNRA	Dissimilatory Nitrate Reduction to Ammonium
DPX	Distyrene, Plasticizer and Xylene
D_W	Diffusion coefficient in water
ε	Porosity
Fe	Iron
Fe^{2+}	Iron(II) ion
H^+	Hydrogen ion
HPTS-TOA	1-hydroxypyrene-tris-3,6,8-octadecylsulfonamide
H_2S	Hydrogen sulfide
K^+	Potassium ion

k_{bio}	Biotransformation rate constant
LED	Light Emitting Diode
LIX	Liquid Ion eXchanger
M_B	Molar weight of the liquid medium
Mg	Magnesium
Mo	Molybdenum
Mn	Manganese
N	Nitrogen
N_2	Dinitrogen
Na^+	Sodium ion
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrogen phosphate
$NaNO_3$	Sodium nitrate
NanoSIMS	Nano secondary ion mass spectrometry
η_B	Viscosity of the liquid medium, B
Ni	Nickel
N_2O	Nitrous oxide
NH_4^+	Ammonium ion
NO	Nitric oxide
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
O_2	Oxygen
φ_B	Solvent association parameter for the liquid medium, B
Pb	Lead
R	Grain radius
R^2	Coefficient of determination
Re	Rhenium
S^{2-}	Sulfide ion
SO_2	Sulfurdioxide
SO_4^{2-}	Sulfate ion
T	Absolute temperature

TDMA	Tridodecylmethylammonium
TOC	Total Organic Carbon
U	Uranium
UV	Ultra Violet
V_A	Solute specific molar volume at normal boiling point for the solute, A
Zn	Zinc

1 Introduction

Nitrogen is not merely an abundant element on earth, making up roughly 80% of the earth's atmosphere, it is also essential for life, and the nitrogen cycle is of great importance to many organisms, human activities and ecosystems. Most of the nitrogen in the atmosphere is in the form of unreactive dinitrogen, N_2, which cannot be utilized by most organisms. Other nitrogen containing compounds are more reactive and can be assimilated by organisms and utilized as building blocks for other compounds or used to generate energy. These reactive nitrogen containing compounds include oxidized and reduced forms of nitrogen such as nitrate, ammonium, amino acids, peptides and proteins - but many other such compounds exist.

While reactive nitrogen containing compounds are essential for life, they are generally in short supply in many ecosystems and as such many organisms are limited by their availability. Their limited availability has also inhibited industrial and agricultural productivity. Up to and including the nineteenth century Europe was dependant on limited supplies of mined nitrogen compounds such as Chile saltpeter and guano for agricultural fertilizers, and industrial production of consumer goods and munitions for military uses. This was the case to such an extent that both food supplies and military security depended on these reserves (Erisman et al., 2008; Sutton et al., 2009).

This widespread dependence was not sustainable, reserves dwindled, and could not be expected to sustain the needs of a rapidly growing population. At this point humans turned their eyes to the skies and the abundant atmospheric store of dinitrogen (Crookes, 1898). Early attempts at fixing nitrogen led to the development of the cyanamide and arc processes, and while they did provide reactive nitrogen forms, they were also unfeasibly expensive in terms of energy consumption (Sutton et al., 2011).

The industrial introduction of the Haber-Bosch process at the beginning of the twentieth century for converting N_2 into ammonia greatly reduced the cost of fixating nitrogen, and meant that the demand for reactive nitrogen compounds from agriculture, industry and military could finally be met (Haber, 1920). Being limited by available reactive nitrogen compounds fast became a thing of the past (Partington, 1925) and in the middle of the twentieth century fossil nitrogen reserves had been replaced by the Haber-Bosch as the main source of reactive nitrogen compounds.

The impact of the Haber-Bosch process on human society and on our world as a whole can hardly be overestimated. Without the nitrogen fertilizers synthesized as a result of its use the human population would not have reached 6 billion people. It has also been estimated that roughly half of those 6 billion people would not be alive without it, and sustaining the predicted growth of the human population without the nitrogen compounds it supplies us with is currently unfeasible (Erisman et al., 2008).

Increased production of nitrogen fertilizers allowed us to modify ecosystems to optimize food production yields, however at first it was done without considering the impact of using agricultural processes with low nitrogen-use-efficiency. Much of the reactive nitrogen has ended up as nitrate pollution in soil and water and emissions of nitrous oxide and ammonia to the atmosphere, resulting in eutrophication, reductions in biodiversity and climate change which threaten our world. Looking at an even grander perspective, the increased production and use of reactive nitrogen compounds has had even more widespread consequences: the polluting nitrogen compounds interact directly with other element cycles in the environment, and by fueling our population growth it also fuels our consumption of other resources such as metals, whose production bring about even more pollution (Sutton et al., 2011).

The impact of fossil fuel combustion is becoming increasingly evident and acknowledged. Likewise, the awareness of the fact that we must take steps to curtail our use of fossil fuels, take steps to limit the environmental impact of the fossil fuels we do use, and deal with the pollution and changes that have already taken place, is growing. In recent times the realization that reactive nitrogen compounds have not just allowed us to feed the world but that their use also has brought with it widespread consequences for us and our surroundings, and that is leading us to take similar steps as for carbon-based pollution when it comes to reactive nitrogen compounds (Blackburn and Sorenson, 1988; Mosier et al., 2004; Söderlund and Svensson, 1976; Stewart and Rosswall, 1982).

Often funding is provided to deal with specific issues relating to reactive nitrogen compounds, such as the need to reduce nitrogen pollution in water, without necessarily keeping the big picture in mind (Sutton et al., 2011). This introduces the risk of researchers focusing on isolated problems, giving less attention to what the consequences of their proposed solutions may be for other ecosystems than the one they are focusing on. Conceivably this can lead

to a situation such as nitrogen pollution in water being converted to nitrogen pollution in air. Thus it is important to focus on solutions which doesn't merely transfer pollution from one area to another, but instead reduce the overall pollution and reduce our need for nitrogen fertilizers.

Nitrogen cycling in terrestrial soil ecosystems has a big potential for impacting other ecosystems. Soil serves many important functions in the nitrogen cycle, including protecting the quality of air and water by filtering, storing, buffering, and transforming reactive nitrogen compounds through biological and chemical processes. These functions come under threat when an excessive nitrogen input decreases biodiversity and changes soil dynamics, both on a macroscopic (Butterbach-Bahl et al., 2011) and a microscopic level (De Vries et al., 2002; Johansson et al., 2004; Streeter, 1988). Nitrogen cycling in soil has been shown to be impacted by the high spatial heterogeneity of soil at the small scale, the fluxes of reactive compounds that permeate it and the microbial communities which inhabit it (Groffman et al., 2009).

1.1 Heterogeneity of soil

The first works on soil heterogeneity were by and large zoological studies investigating how macro organisms influence the environment (Aller, 2001, 1982; Buckland, 1835; Cunningham and Ramage, 1888; Dapples, 1942; M'Intosh, 1894; Watson, 1890).

Then, more than 60 years ago it was suggested that the presence of metal sulfides within oxidized marine sediments was due to the presence of niches where a high localized content of organic matter creates reducing conditions (Emery and Rittenberg, 1952). This was suggested again years later (Emery et al., 1963; Hallberg, 1968), and in 1977 Jørgensen expanded upon the concept of reduced niches within an oxic environment. He demonstrated that bacterial sulfate reduction took place in an overall oxidized environment, and furthermore that it took place within anoxic niches with a diameter of 50 to 200µm, which explained the presence of sulfate reducing and sulfide oxidizing bacteria in the system. The study furthermore suggested that such niches could be of importance for a wide range of metabolic and diagenetic processes (Jørgensen, 1977).

The concept of microscale niches and our knowledge of soil heterogeneity has been expanded significantly since then. Soil is characterized by both physical and temporal heterogeneities across all scales, from nm to km

(Young and Ritz, 2000). Small scale features such as pore pathways influence processes ranging from water retention (Vogel, 2000) to plant productivity (Stirzaker et al., 1996) and further on to greenhouse gas emissions (Arah and Vinten, 1995).

Looking at molecular scales soil constituents such as clay platelets are dominated by electrostatic and van der Wall forcers. At larger scales soil microorganisms exuding a matrix that, along with plant roots, bind particles together (Young and Crawford, 2004).

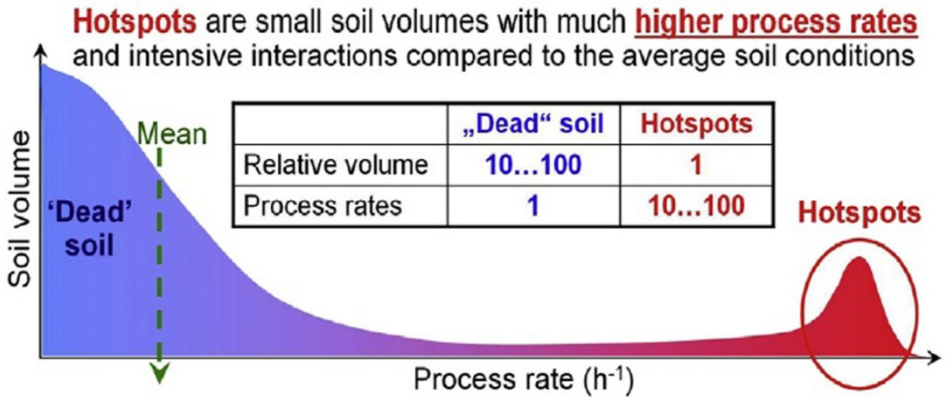

Figure 1. Microbial hotspots in soil: Hotspots are small soil volumes with much higher process rates and intensive interactions compared to average soil conditions. The Table inset represents the relative volume and process rates in the hotspots and bulk soil. "Mean" represents the weighted average process rates by soil mixing. From (Kuzyakov and Blagodatskaya, 2015).

Soil microorganisms tend to form colonies and biofilms (Ekschmitt et al., 2005; Hodge et al., 1998), and even though there can be 10^7 to 10^{12} microbial cells in one gram soil (Watt et al., 2006), they are restricted to a fraction of the total volume, as little as 1% (Young et al., 2009). As a result of this ecologically relevant biogeochemical processes mainly take place in microbial hotspots. These have been defined as small soil volumes with much faster process rates and much more intensive interactions between element pools compared to average soil conditions, to such an extent that they are relevant on higher scales, as shown in figure 1 (Kuzyakov and Blagodatskaya, 2015).

But the microorganisms are not only limited by their spatial distribution, they are also limited by the availability of resources such as carbon and physical

parameters such as temperature (Hodge et al., 2000). This limits their activity and often reduces it to a state of dormancy (Blagodatskaya and Kuzyakov, 2013). However, when the limitations are temporarily removed the microorganisms become active during what has been named a hot moment. These are defined as short-term events or sequences of events that accelerate microbial processes as compared to the average rates (Kuzyakov and Blagodatskaya, 2015).

These definitions are based on dynamic properties, the intensity of microbial processes, and underline that both hotspots and hot moments are dynamic in nature. It should be noted that while hot moments are events that take place in hotspots, hotspots are not limited to existing during hot moments. Once a hot moment ends a hotspot can return to a state of dormancy if the conditions allow it.

It is difficult to accurately estimate the size of hotspots in a given system, but the impact of individual microbial cells is too low to be of note on higher scales (Kuzyakov and Blagodatskaya, 2015), so the term hotspot should be reserved for microcolonies, biofilms and larger microbial communities (Panikov, 2010). This means that the minimal size of a microbial hotspot is in the vicinity of a few μm (Dechesne et al., 2003; Eickhorst and Tippkötter, 2008; Raynaud et al., 2014). At the other end of the scale visualizations of oxygen consumption, pH and redox changes give values up to 10mm (Blossfeld, 2013; Rudolph et al., 2013; Schmidt et al., 2010). Likewise it can be difficult to estimate the duration of hot moments, but looking at hot moments induced by an increase in available carbon they range from a day or less (Jones et al., 2005; Pausch and Kuzyakov, 2011) to more than a month (Bastian et al., 2009; Poll et al., 2010; Spohn and Kuzyakov, 2014), based on the form of carbon that is made available to the microorganisms. The described spatial extents and temporal durations in different soil fractions are shown in figure 2.

Hotspots are not merely characterized by high activity, as per figure 1, they also have a higher microbial diversity compared to individually scattered microbial cells in soil (Lee et al., 2013). Additionally the microbial composition of a given hotspot may change when environmental conditions change (Remenant et al., 2009; Schmidt and Eickhorst, 2014). However, this high, plastic diversity does not necessarily result in a change in hotspot function, as functional redundancy lead to high similarity between hotspots differing in microbial community structure (Ruamps et al., 2013), at least

unless the plant diversity in the ecosystem drops significantly (Loranger-Merciris et al., 2006; Sanaullah et al., 2011).

The high involvement of hotspots and hot moments in environmental processes such as carbon, nitrogen, sulphur and metal cycling (Groffman et al., 2009; Hansel et al., 2008; Kuzyakov and Blagodatskaya, 2015; Widerlund and Davison, 2007) makes elucidating their inner workings of high importance if we are to fully understand these processes. But due to the limited spatial size of hotspots, the limited duration of hot moments and the extensive heterogeneity inside and surrounding them in the form of community spatial structure and gradients created by diffusive limitation, it is a difficult task to study these small scale phenomena. It cannot be accomplished by solely looking at large scale heterogeneity, and nor can it be done with techniques that mix the hotspots with bulk soil (Ruamps et al., 2011).

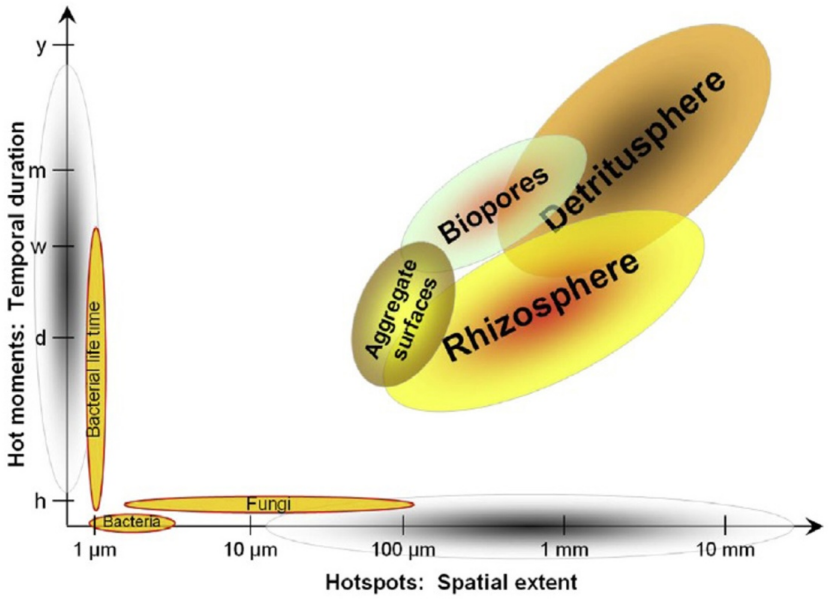

Figure 2. Spatial and temporal scales of microbial hotspots in soil. The allocation of areas corresponds to the size and duration of microbial hotspots and hot moments, but not to the size of the properties themselves: e.g. aggregate surfaces may exist years and decades, but the duration of hotspots on the aggregate surfaces is limited by days. Logarithmic Y scale corresponds to h: hours, d: days, w: weeks, m: months, y: years. The intensity of grey on shaded areas on X and Y axis represents schematically the spatial and temporal probability of microbial hotspots. From (Kuzyakov and Blagodatskaya, 2015).

If we are to get a good picture of the impact of physicochemical soil heterogeneity on microbial processes then we need both tools for measuring

gradients and spatial structure, and ways of estimating the processes that work on the scale of hotspots, such as diffusive limitation, and their importance for ecosystem functioning.

1.1.1 Estimating the impact of mass transfer kinetics on microbial processes

Part of the heterogeneity of soil stems from the aggregative nature of soil, and the activity of microorganisms depends on physicochemical phenomena which control the availability of nutrients. Modeling efforts have yielded a number of dimensionless parameters for assessing the impact of mass transfer kinetics on biotransformation rates. Three such parameters, the Damköhler numbers, can be used to quantify the impact of advection, sorption, and diffusive limitation on microbial processes.

The first Damköhler number, Da_1, can be used to quantify if advection limits microbial processes, it is defined as:

$$Da_1 = \text{biodegradation rate / advection rate}$$

If $Da_1 > 1$, then advection takes place much slower than biotransformation, and increasing the flow rate can increase the overall biotransformation, but only as long as Da_1 remains larger than 1 (Sims and Overcash, 1983).

Soil-water systems are dominated by surfaces to which microorganisms attach, and the microorganisms are excluded from entering all parts of the soil aggregates due to their size. This means that soluble electron acceptors and donors in these areas must first diffuse through the pore water to the surface of the aggregates before they become available to the microorganisms (Ramaswami and Luthy, 2002). The second Damköhler number, Da_2, shows if biotransformation is limited by desorption from soil aggregates, and is defined as:

$$Da_2 = \text{biodegradation rate / external mass transfer rate}$$

If $Da_2 > 1$, then the desorption rate limits biotransformation, and biotransformation may be increased by increased mixing (Ramaswami and Luthy, 1997; Ramaswami et al., 1997; Seagren et al., 1993).

The third Damköhler number, Da_3, sometimes referred to as the Thiele modulus, is defined as

$$Da_3 = \text{biodegradation rate / diffusion rate}$$

, and can be calculated using:

$$Da_3 = k_{bio} \cdot R^2 / D_{eff}$$

, where k_{bio} is the biotransformation rate constant, R is the aggregate radius, and D_{eff} is the effective intra-aggregate diffusion coefficient that accounts for sorption-retarded diffusion within aggregates. If $Da_3 > 1$, then intra-aggregate diffusion limits biotransformation (Chung et al., 1993; Ramaswami and Luthy, 1997; Ramaswami et al., 1997).

The Damköhler numbers are most often used in the study of pollution and bioremediation, but since they are based on parameters such as advection rate, diffusion coefficients and aggregate sizes they can be applied to a wide range of 'chemical compound-soil system' scenarios.

One such scenario is the impact of heterogeneity, in the form of redox gradients created by diffusive limitation, on microbial soil processes such as the nitrogen cycle, a group of processes that has been shown to be dominated by microbial hotspots (Groffman et al., 2009).

1.2 The nitrogen cycle

Nitrogen cycling in terrestrial settings such as forests and agriculture is driven by microbial and plant processes, and physico-chemical processes such as diffusion, leaching, volatilization and emission which displace nitrogen locally and on larger scales (Erisman et al., 2008; Galloway, 2003). If we are to understand the impact of increased nitrogen availability on ecosystems and be able to predict which changes will occur, then we must have detailed knowledge of the processes that make up the nitrogen cycle (figure 3).

The nitrogen cycle is intimately connected to the carbon cycle: as one of the building blocks of amino acids and nucleic acids nitrogen is a part of all proteins and hereditary material, and a large part of nitrogen in primary producers is put to use in photosynthesis as a building block of chlorophyll, the pigment that is responsible for capturing light energy (Evans, 1989). It is primarily made up of microbial processes in soils, sediments and water (Seitzinger et al., 2006). Part of these processes take advantage of nitrogen's

wide range of oxidations stages, from -III in NH_4^+ to +V in NO_3^-, to either gain energy from oxidizing nitrogen or utilized oxidised nitrogen compounds as electron acceptors for anaerobic growth; these processes include denitrification, dissimilatory nitrate reduction to ammonium (DNRA), anaerobic ammonium oxidation and nitrification.

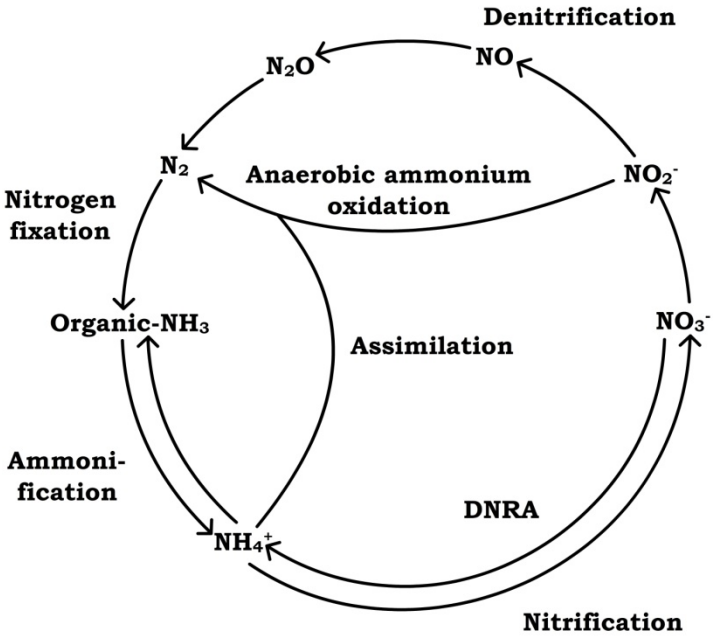

Figure 3: Nitrogen can have a wide range of oxidation stages, exemplified by compounds involved in the nitrogen cycle: -3 in NH_4^+, 0 in N_2, +1 in N_2O, +2 in NO, +3 in NO_2^-, +5 in NO_3^-. The reactions composing the nitrogen cycle make use of this to gain energy from oxidizing nitrogen or utilized oxidised nitrogen compounds as electron acceptors for anaerobic growth.

Land use and ecosystem type both have a big impact on nitrogen cycling and storage. Agricultural settings are largely dominated by the use of nitrogen fertilizers and the removal of crop, while primarily natural system nitrogen cycling is to a large extent decided by landscape and climatic conditions, and nitrogen inputs in the form of biological nitrogen fixation and nitrogen deposition. Shrublands (ecosystems characterized by vegetation dominated by shrubs, grasses and other plants of limited height) and wetlands (terrestrial ecosystems characterised by permanent or seasonal saturation with water) are primarily nitrogen limited systems due to shortages in available nitrogen for the former and high losses as a result of denitrification for the latter. In this

context nitrogen limited means that plant production is hampered due to shortages in available nitrogen. Human use such as cattle grazing has depleted the nitrogen stores of shrublands over centuries, and they may also have properties such as high sand content and low organic matter content which gives the environment reduced ion exchange capacities and poor nitrogen retention, respectively. In recent times some of these areas are being exposed to high rates of nitrogen deposition and exhibit signs of being nitrogen saturated such as reduction in biodiversity and nitrate leaching (Schmidt et al., 2004). While tropical forests tend to be nitrogen rich systems, temperate forests are naturally nitrogen limited ecosystems - this has however changed somewhat in recent times as a result of nitrogen input via atmospheric deposition (Butterbach-Bahl et al., 2011) and leaching from nearby animal farms (Dragosits et al., 2002). This increased input has led to significant nitrate leaching and nitrous oxide emission (Dise et al., 2009; Pilegaard et al., 2006).

Nitrate is a key compound in the nitrogen cycle, and its fate, and thus also the fate of the ecosystem nitrogen balance, is in many ecosystems decided by the balance between two dissimilatory nitrate reduction processes: denitrification, in which nitrate is converted to the gaseous compounds dinitrogen and nitrous oxide, and DNRA. While both processes bring about the reduction of nitrate, their impact on the ecosystem is radically different. In the following the nitrogen cycle will primarily be considered in terms of nitrogen inputs to terrestrial systems.

1.2.1 Nitrogen inputs to ecosystems

Biological nitrogen fixation used to be the major source of reactive nitrogen compound production in preindustrial times, and this is still the case for some unspoiled ecosystems (Cleveland et al., 1999). Despite being needed for all life and being the major constituent of our atmosphere dinitrogen cannot be used directly for biological functions by living organisms. First the strong triple bond between the two nitrogen atoms must be broken. In nature this is done through biological nitrogen fixation, and industrially through the Haber-Bosch process - in both cases the overall stochiometry of the reaction is $N_2 + 3\ H_2 \rightarrow 2\ NH_3$.

In soil biological nitrogen fixation is performed by heterotrophic soil bacteria and as a result of a symbiotic relationship between a bacterium infecting the roots of a plant, often a legume, where the plant supplies nutrients to the bacterium and in return gets reactive nitrogen compounds (Eskew et al.,

1981). The capacity of biological nitrogen fixation is high in many systems, and can be up to 200 kg N ha^{-1} yr^{-1} (Carlsson and Huss-Danell, 2003; Cleveland et al., 1999; Smil, 1999), being able to bring the supply of reactive nitrogen to such a level that the system instead becomes limited by other resources such as phosphorous (Vitousek et al., 2002).

Nitrogen is also deposited to ecosystems from the atmosphere as precipitation, gasses and particles. Precipitation can contain dissolved HNO_3, NH_3, HNO_2, and organic nitrogen compounds; the gasses include NH_3, HNO_3 and NO_2, and the particles most often considered in dry deposition are composed of $(NH_4)_2SO_4$ and NH_4NO_3 (Hertel et al., 2011). For shrublands and similar ecosystems with short vegetation precipitation is the primary source of nitrogen input from the atmosphere, and in Europe it normally falls in the range of 3-30 kg N ha^{-1} yr^{-1}. Forests, on the other hand, are very efficient sinks for nitrogen containing water soluble gasses and for particles, and nitrogen deposition can here be twice as high as for shrubland, falling in the range of 5-60 kg N ha^{-1} yr^{-1} (Dise et al., 2009), making forests most exposed to nitrogen deposition from the atmosphere (Fowler et al., 2005).

The nitrate fraction of the atmospheric nitrogen input originates from nitrogen oxides created as a result of the burning of fossil fuels, and its contribution to the nitrogen to terrestrial ecosystems can be up to 15 kg N ha^{-1} yr^{-1} (Fagerli and Aas, 2008). However there can be high local variability, and consequently some areas can receive large amounts of nitrate (Dragosits et al., 2002).

The use of synthetic fertilizers has increased considerably over the past half century; in 1950 the global consumption of nitrogen fertilizer per year was equal to 4 Mt nitrogen, this increased to 32 Mt in 1970 and in the 1990s it was greater than 80 Mt, and in recent years it has decreased slightly in some areas as a reaction to regulations imposed to limit leaching of nitrate and increases in the price of fertilizer (Roy and Hammond, 2004). The average fertilizer and input to European agricultural soil has been as high as 123 kg N ha^{-1} yr^{-1} (van Egmond et al., 2002).

In comparison to the above values a growing temperate forest performs nitrogen cycling in the form of root and leaf production and turnover in the range of 60-100 kg N ha^{-1} yr^{-1} (Kreutzer et al., 2009), and has a net demand of nitrogen in the vicinity of 5-10 kg N ha^{-1} yr^{-1} (Scarascia-Mugnozza et al., 2000). At the same time microbial nitrogen cycling in the form of mineralization, nitrification and immobilization has been estimated to being

as high as 1000 kg N ha^{-1} yr^{-1} in a Fagus sylvatica forest in Germany recieving high amounts of nitrogen through precipitation (Corré et al., 2003).

1.2.2 Nitrate removal processes

Several microbial processes act on soil nitrate. Denitrification converts it to dinitrogen with nitrite, nitric oxide and nitrous oxide as intermediary steps. DNRA converts it to ammonium. Another process, anaerobic ammonium oxidation (anammox), can utilize intermediary products from the two other processes, transforming nitrite and ammonium into dinitrogen.

Microbial processes are only part of the story, however physical processes also play their part. Nitrate itself is highly mobile in soils and a combined nitrate input and production in excess of the requirements of plants and microorganisms will be transported though the soil and leached to other environments.

Nitrogen deposition below 10 kg N ha^{-1} yr^{-1} rarely leads to increased nitrate leaching compared to the average for the ecosystem, but it always occur when it is above 25 kg N ha^{-1} yr^{-1}. Between these values nitrate retention can range from 0 to 100%. Systems in which biological processes are limited by nitrogen generally have a high retention and systems that are not limited have a low retention (Butterbach-Bahl et al., 2011).

When looking at agricultural soils increasing the rate of nitrogen fertilizer above the crop nitrogen demand leads to a rapid increase in leaching (Vinten et al., 1994), but it can also be impacted by rainfall and soil management. Ploughing down plants and reseeding them can be used to reduce nitrate leaching, increasingly so if the process is repeated year after year (Shepherd et al., 2001), and applying raw sludge instead of digested sludge likewise reduce leaching (Misselbrook et al., 1996) This is presumably caused by repeated treatment making the soil capable of utilizing more nitrogen, and the raw sludge containing more complex nitrogen compounds than the digested sludge, leading to a slower release of accessible nitrogen and less leaching.

In the following only the microbial processes which directly involve nitrate in soil are covered.

1.2.3 Denitrification

Denitrification is the microbially catalyzed dissimilatory reduction of nitrate, nitrite, nitric oxide or nitrous oxide to nitrous oxide and dinitrogen. The pathway is found in both bacteria, fungi and archaea (Hayatsu et al., 2008; Zumft, 1997). It is primarily performed by facultatively anaerobic bacteria

that usually respire both oxygen, but turn to nitrogen oxides under anaerobic conditions. While the enzyme catalyzing the reaction is usually expressed under oxygen-limiting conditions, some bacteria have been shown to be able to perform aerobic denitrification (Bateman and Baggs, 2005). Denitrifiers have been identified in more than 60 bacterial genera from the phyla Aquificae, Deinococcus-Thermus, Firmicutes, Actinobacteria, Bacteroides, and Proteobacteria (Zumft, 1997), representing roughly 5% of the total soil microbial community (Philippot et al., 2007; Wallenstein et al., 2006). Aditionally, some fungi (Shoun et al., 1992; Tanimoto et al., 1992) and archaea (Philippot, 2002) can perform denitrification.

Microbial denitrification is considered to be the main pathway of nitrogen loss in terrestrial ecosystems, having been estimated to account for 40% of the global nitrogen addition to terrestrial ecosystems (Seitzinger et al., 2006; Van Breemen et al., 2002). Denitrification rate estimates for different ecosystems vary with soil properties and management (Barton et al., 1999; Hofstra and Bouwman, 2005), and also with the method used to quantify nitrogen losses, with mass balance based estimates twice as high as soil-core based ones (Groffman et al., 2006; Hofstra and Bouwman, 2005). In general the denitrification rate is estimated to be around 2 kg N ha^{-1} yr^{-1} for forests and 13 kg N ha^{-1} yr^{-1} for agricultural soils (Barton et al., 1999), and there are large variations from one area to another (Seitzinger et al., 2006).

The supply of nitrate is known to be a strong control on denitrification rates, however there has been found little correlation between nitrate concentration and denitrifier abundance based on DNA hybridization (Mergel et al., 2001). Instead soil denitrifying communities are moulded by the availability of carbon, pH and the range of moisture and carbon they experience, and also by predation by fauna and viruses and ecosystem disturbances such as freeze/thaw, wetting/drying, fire and physical disruption (Wallenstein et al., 2006).

The process of reducing nitrate to dinitrogen involves four sequential enzymatically catalyzed reductions (Philippot et al., 2002), as shown in figure 4. The first step is the reduction of nitrate to nitrite is catalyzed by two homologous enzymes, Nar and Nap. The former is membrane-bound and the latter periplasmatic bound. Both of these enzymes are also present in non-denitrifying bacteria such as nitrate respirers and bacteria performing DNRA, and for this reason they are not used much for characterizing denitrifiers (Chèneby et al., 2004, 2003; Philippot et al., 2002).

The next step is reduction of nitrite to nitric oxide catalyzed by a nitrite reductase, of which there are two evolutionary unrelated forms, a nitrite reductase incorporating copper encoded by *nirK*, and one utilizing cytochrome *cd1* encoded by *nirS* (Braker et al., 1998). These genes are the most commonly used markers for denitrifying bacteria. Then follows reduction of nitric oxide to nitrous oxide catalyzed by nitric oxide reductase encoded by *norB*, this enzyme creates the bond between the two nitrogen atoms. *nosZ* encodes the nitrous oxide reductase which catalyzes the final step in denitrification: the reduction of nitrous oxide to dinitrogen. Like *nirK* and *nirS*, *nosZ* has been used widely to characterize denitrifiers (Scala and Kerkhof, 1998). While many bacteria contain all genes necessary to perform denitrification, some only contain a partial suite. For these denitrification is restricted to being a community effort, since they have to work with other community members in order to achieve complete denitrification (Zumft, 1997).

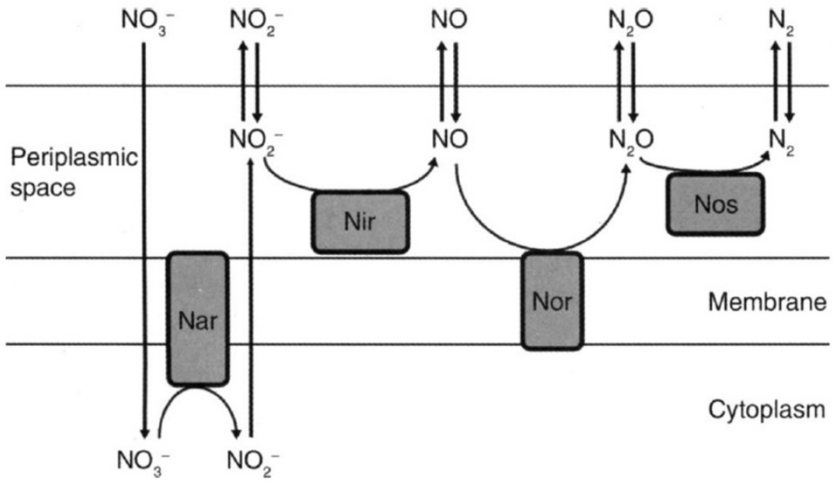

Figure 4: Sequential reductive pathway of denitrification showing the location of enzymes relative to the cytoplasmic membrane: Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase. From (Wallenstein et al., 2006).

There have been studies who have found a correlation between the abundance of denitrification genes and soil denitrification potential (Rich et al., 2003) and the ratio between denitrification products (Chèneby et al., 1998). But the link between community structure and function is not always present. One study found that while *nosZ* profiles and the activity of nitrous oxide reductase varied among different soils, they did not follow the same pattern, suggesting that the two were not linked (Rich and Myrold, 2004). Another study found that changes in soil nitrous oxide reductase activity effected by

experimental manipulations didn't result in a changed *nosZ* profile (Boyle et al., 2006). Thus the relative activity of denitrification enzymes may sometimes be affected by denitrifier composition, but at other times activity is primarily decided by environmental factors.

Microbial denitrification is not the sole possible denitrification pathway: the presence of Fe^{2++} under alkaline pH can induce chemo-denitrification (Samarkin et al., 2010; van Cleemput, 1998).

1.2.4 Dissimilatory nitrate reduction to ammonium

DNRA is the anaerobic conversion of nitrate to ammonium, and is also known as fermentative ammonification, fermentive nitrate reduction and nitrate ammonification. The process was studied extensively in anaerobic sludge and sediments where it can be very active (Ambus et al., 1992; Bonin, 1996; Nijburg et al., 1997; Tiedje et al., 1982), but it has later been found to be environmentally relevant in both tropical forests (Silver et al., 2001) and temperate soils (Müller et al., 2007, 2004).

What makes DNRA exceptionally interesting is that it bypasses denitrification, provides ammonium for immobilization in microorganisms and plants and reduces the pool of available nitrate that can leach out of the soil or be converted to gasses which may escape the system. This means that a better understanding of DNRA and the factors that control it can be a path towards increasing ecosystem nitrogen retention (Silver et al., 2001). Several groups of bacteria have been found to be capable of performing DNRA , ranging from members of the Bacteroides (Mohan et al., 2004) to Gamma-, Delta- and Epsilonproteobacteria (Smith et al., 2007).

The environmental conditions that influences DNRA are soil oxidation state and the carbon/nitrate ratio. DNRA has been reported to occuring under more anoxic conditions than denitrification, but it has also been shown that DNRA can be less sensitive to variable redox conditions(Page et al., 2003; Takaya, 2002; Yin et al., 2002) and oxygen than denitrification (Fazzolari et al., 1998). An incubation of soil aggregates under various oxygen levels and carbon additions, but constant nitrate concentration, showed that the impact of oxygen level on DNRA depended on the carbon/nitrate ratio, and concluded that the balance between denitrification and DNRA was primarily decided by the abundance of carbon in the system (Fazzolari et al., 1998). This is supported by another study which found that DNRA only took place in significant amounts when the carbon/nitrate ratio was above 12 (Yin et al., 1998). The effect of carbon/nitrate ratio has been argued to be due to

experimental modifications of the carbon/nitrate ratio resulting stimulating microbial activity, which leads to an increased consumption of oxygen, and thus an altered soil oxidation state which leads to increased DNRA (Matheson et al., 2002).

Like for denitrification the first step in DNRA is the reduction of nitrate to nitrite by a nitrate reductase, but where denitrification is catalyzed by a series of four enzymes, the remaining steps of DNRA is catalyzed by the NrfA nitrite reductase in the periplasm, encoded by the *nrfA* gene, which converts nitrite to ammonium.

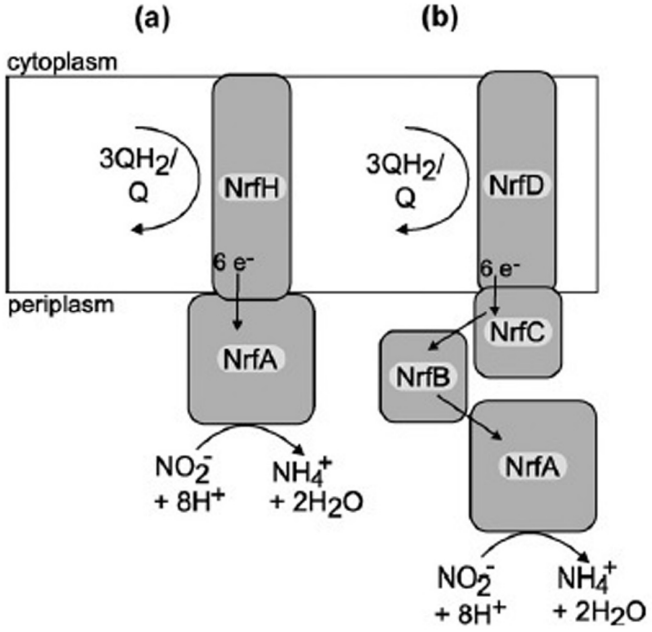

Figure 5. Respiratory chain in dissimilatory nitrate reduction to ammonium. The nitrite reductase is part of a complex with one (a) or more (b) other subunits, depending on the species. From (Kraft et al., 2011).

NrfA functions as part of a complex with one or more other subunits, depending on the species, but as shown in figure 5 the overall reaction remains the same, and it is assumed that it has enzyme-bound nitric oxide and hydroxylamine as intermediary steps (Cruz-García et al., 2007; Vermeiren et al., 2009). Using *nrfA* as a marker gene is difficult as few sequences are available, and DNRA is phylogenetically widespread in nature. *nrfA* primers have been designed based on the alignment of six *nrfA* sequences including the ones from *Escherichia coli* K-12, *Sulfurospirillum deleyianum* and *Wolinella succinogenes*, and have been used to detect sequences from several

bacterial species, primarily Bacteroides. The primers did however also detect a gene encoding a c-type cytochrome, unrelated to *nrfA* (Mohan et al., 2004).

Although most bacteria capable of performing DNRA contain both the core nitrite reductase and a nitrate reductase, some sulphate reducing bacteria lack the nitrate reductase and can only use nitrite (Dannenberg et al., 1992; Mitchell et al., 1986). There also exists a dissimilatory version of DNRA catalyzed by Nir, an enzyme composed of two subunits encoded by *nirB* and *nirD*, and located in the cytoplasm. Unlike the periplasmatic Nrf, NirBD is not involved in respiratory DNRA, instead it acts as an electron sink and detoxifies nitrite created during nitrate respiration (Cabello et al., 2012).

1.2.5 Thermodynamics of nitrate reduction

Oxidation of biomass yields the highest amount of energy when oxygen is used as an electron acceptor, however coupling the oxidation of biomass (in the following represented by glucose) with reduction of nitrate to dinitrogen or ammonium gives almost as much energy per mol electron donor:

Aerobic respiration:

$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$

($\Delta G^{o'}$ = -2870 kJ per mol glucose)

Denitrification:

$5\ C_6H_{12}O_6 + 24\ NO_3^- + 24\ H^+ \rightarrow 30\ CO_2 + 42\ H_2O + 12\ N_2$

($\Delta G^{o'}$ = -2670 kJ per mol glucose)

DNRA:

$C_6H_{12}O_6 + 3\ NO_3^- + 6\ H^+ \rightarrow 6\ CO_2 + 3\ H_2O + 3\ NH_4^+$

($\Delta G^{o'}$ = -1870 kJ per mol glucose)

However when the energy is calculated per mol electron acceptor, $\Delta G^{o'}$ becomes -478, -556 and -623 kJ per mol oxygen/nitrate for aerobic respiration, denitrification and DNRA, respectively. These values mean that denitrification should be the preferred nitrate reduction mechanism when

microorganisms are limited by the available organic carbon, and that DNRA should be preferred when they are limited by nitrate, which has been confirmed experimentally (Otte et al., 1999; Schulz and Jørgensen, 2001; Schulz et al., 1999).

1.2.6 Other processes

Both ammonium and nitrate can be taken up by bacteria, fungi and plants to be immobilized in the ecosystem. Since both nitrogen and carbon are core building blocks of most cell constituents, their uptake by organisms is correlated to each other. While many sea organisms are seen to follow the Redfield ratio of 53C:8N (Redfield, 1934), things are not so simple for most terrestrial organisms such as plants (Sterner and Elser, 2002), however nitrate immobilization has been shown to be dependant on available carbon (Booth et al., 2005; Nishio et al., 2001; Trinsoutrot et al., 2000), and the same is the case for ammonium (Recous et al., 1990; Rice and Tiedje, 1989).

Denitrification isn't the only process capable of producing dinitrogen, anammox can convert ammonium and nitrite into dinitrogen (Hayatsu et al., 2008), but while bacteria capable of performing anammox have been found in soil, the process has not yet been shown to be significant here (Butterbach-Bahl et al., 2011; Hayatsu et al., 2008).

1.3 Global change and the nitrogen cycle

The use of nitrogen fertilizers and nitrogen deposition from the atmosphere can directly alter nutrient ratios in soil and plants, damage vegetation, cause eutrophication, and increase soil acidity through a complex web of nitrogen transformations as shown in figure 6, and has been recognised as one of the key threats to global biodiversity (Butterbach-Bahl et al., 2011; Hertel et al., 2011; Sutton et al., 2011). While many would agree that having diverse ecosystems has value in itself, many important functions can be described directly to biodiversity, such as increasing an ecosystems stability and resilience towards stress and serving as a library of genetic diversity from which we may discover future industrial, medical, and food products (Ehrlich and Ehrlich, 1992; Tilman and Downing, 1994; Tilman et al., 1996). As a result of nitrogen limiting productivity in many terrestrial ecosystems, some of the environments most sensitive to eutrophication induced by increased availability of reactive nitrogen compounds are those with low soil nitrogen levels and populated by stress tolerant species who wont be able to compete with species better able to benefit from increased nitrogen availability (Bobbink et al., 1998). Such ecosystems include forests with nutrient poor

soils where evidence has been found for both changes in biomass (Nellemann and Thomsen, 2001) and ground flora composition has been related to increased availability of nitrogen (Pitcairn et al., 1998).

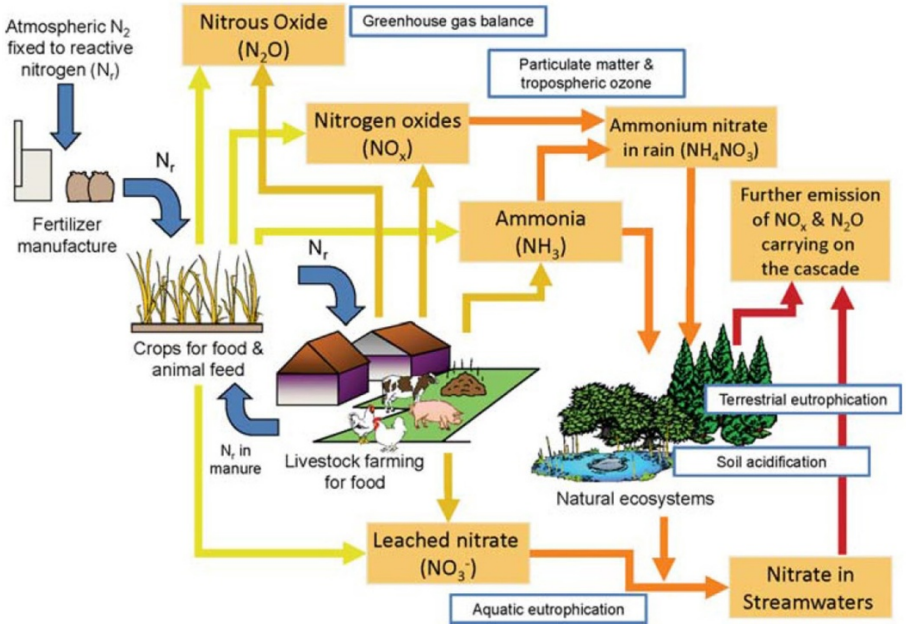

Figure 6: Losses, transformations and effects of reactive nitrogen (N_r) fertilizers in the environment. From (Sutton et al., 2011).

1.4 Motivations and objectives of the PhD study

The terrestrial nitrogen cycle is intrinsically linked with other element cycles and is of great importance to many ecosystems. The processes that constitute the nitrogen cycle are associated with small scale heterogeneity in the form of microbial hotspots and hot moments. Consequently it is imperative that the nitrogen cycle is studied at small scales and in relation to physical processes that are relevant here, such as electron donor/acceptor fluxes influenced by diffusive limitation, something which is currently underexplored in terms of nitrogen cycling (Groffman et al., 2009).

In order to increase the knowledge of nitrogen cycling in terrestrial systems the PhD study has taken a three pronged approach:

1. Develop a framework for imposing a quantifiable degree of diffusive limitation on a system and use it to investigate the impact of diffusive carbon limitation on nitrogen cycling in terrestrial systems, focusing on nitrate metabolism in soil.

2. Review the use of microscale techniques in environmental studies, both relating to nitrogen cycling and to other processes, focusing on microsensors and planar sensors with microscale resolution
3. Develop a tool for studying two-dimensional nitrate distributions at microscale resolution.

For the first part the study, I needed to (Papers I & II):

- identify a terrestrial system in which both DNRA and denitrification takes place.
- use the system to study the impact of the amount of available electron donor on DNRA and denitrification.
- develop and implement a protocol for experimentally imposing several quantifiable degrees of diffusive limitation on the system.
- utilize the identified system and developed protocol to investigate if modulating diffusive carbon limitation impacts nitrogen cycling

For the second part the study, I needed to (Paper III):

- identify and compare microscale techniques available to environmental studies
- create a comprehensive resource describing available microscale techniques, providing key performance characteristics and information on accessibility.

For the third part the study, I needed to (Papers IV & V):

- use the resource created in the second part to identify the need for a tool for studying two-dimensional nitrate distributions at microscale resolution.
- develop the tool, a nitrate sensitive planar optode.
- create and optimize protocols for using the tool.

2 Denitrification versus dissimilatory nitrate reduction to ammonium

Until recent times the study of anaerobic nitrate reduction in soils focused on denitrification (Cole, 1990), which in many ecosystems is the dominant nitrate removal pathway (Baker and Vervier, 2004; Forshay and Stanley, 2005).

It has been shown that the input of reactive nitrogen to the land in the form of fertilizer, atmospheric deposition and nitrogen fixation is a dominant flux in the global nitrogen cycle, and furthermore that most of this nitrogen disappears in the terrestrial part of the cycle (Seitzinger et al., 2006). While we do have some insight into the factors that influence denitrification, the availability of nitrate, organic carbon, and oxygen, we know little about the complex regulation of these factors that results in the formation of hotspots with limited temporal persistence, but big impact on overall nitrate reduction activity (McClain et al., 2003). The distribution of hotspots is dependant on soil type (Cambardella et al., 1994), plant biomass (Robertson et al., 1997) and microtopology (Bruland et al., 2006), which impacts the distribution of organic matter and water (Hafner and Groffman, 2005). But while knowledge of these factors make it possible to estimate nitrate reduction at a landscape scale, it remains challenging to study hotspots and the processes associated with them at the small scales where they exist and take place.

While potentially important environmental controls for deciding the outcome of the competition between denitrification and DNRA are known to include carbon/nitrogen ratio (Schmidt et al., 2011; Strohm et al., 2007; Tiedje et al., 1983; Tugtas and Pavlostathis, 2007), pH (Schmidt et al., 2011), nitrite versus nitrate concentration (Dong et al., 2011, 2009; Schmidt et al., 2011), available carbon (Akunna et al., 1993; Tugtas and Pavlostathis, 2007) and temperature (Dong et al., 2011; Ogilvie et al., 1997), the interplay between the factors is very complex as a result of the heterogeneity of the microbial communities and the environments they inhabit.

Many studies trying to elucidate the primary controls have focused on the ratio between carbon and nitrogen in the system. Most found that a high electron donor/acceptor ratio led to increased DNRA (Fazzolari et al., 1998; Silver et al., 2005; Smith, 1982) however there is also a study that found no connection between the carbon/nitrogen ratio and denitrification versus DNRA (Sotta et al., 2008). A recent study utilizing multiple parallel long-

term incubations of nitrate-respiring communities compared the impact of the potential environmental controls and found that the key factors was the carbon/nitrogen ratio, the supply of nitrite relative to nitrate, and the microbial generation time (Kraft et al., 2014). They also noted that the denitrifying and ammonifying populations were competing for the same electron donors, and that the selective forces seemed to work directly on the nitrite reductases in the two pathways. NrfA, the nitrite reductase in DNRA, requires six electrons per nitrite, and Nir, the nitrite reductase in denitrification, only require a single electron per nitrite, and consequently the latter has an advantage when there is a low supply of carbon, the electron donor. This is also in line with the thermodynamics of denitrification and DNRA presented earlier. NrfA also had a lower affinity for nitrite than Nir, giving denitrification the edge when nitrite was available as electron acceptor. When the generation time was low NrfA could keep up with Nir, and ammonification outcompeted denitrification. This is illustrated in figure 7.

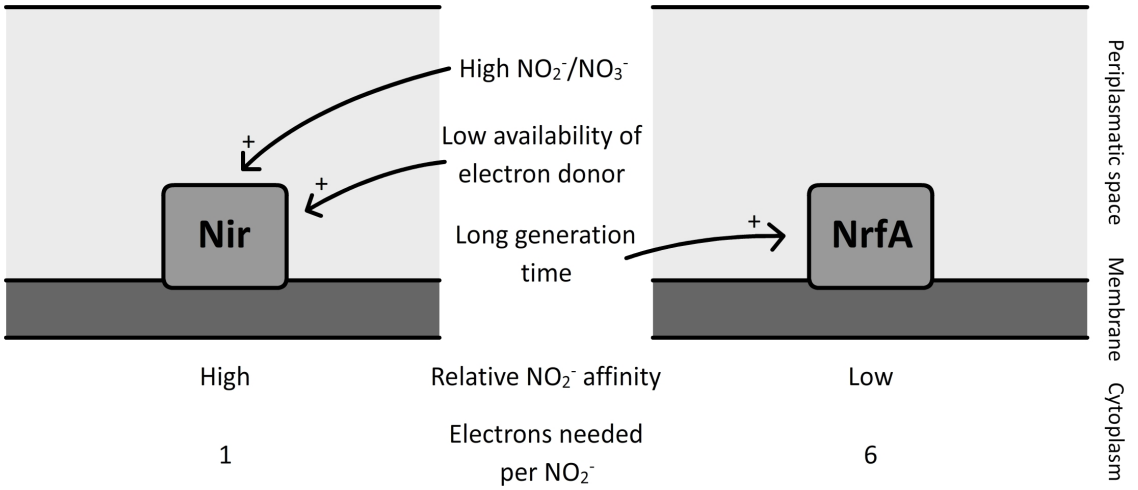

Figure 7 - Parameters influencing the competition between denitrifying and ammonifying populations due to their impact on the nitrite reductases Nir (denitrification pathway) and NrfA (DNRA pathway). Arrows indicate which nitrite reductase is favoured by a given parameter.

To identify a terrestrial system in which both DNRA and denitrification takes place I utilized an array of column microcosms with different amounts of organic matter from *Fagus sylvatica* soil from Dyrehaven in Northern Zealand, Denmark. The columns were packed with increasing amounts of soil (0, 0.3, 3, 30, 300g) as shown in figure 8, and fed a solution of nitrate which

has been sparged with dinitrogen to remove oxygen. The effluent was then analyzed to study the impact of the amount of available electron donor on DNRA and denitrification.

As per figures 9 nitrate profiles for the five columns containing soil were similar to each other, and were seen to have four distinct phases. The columns which had been packed with relatively small amounts of organic matter had initially very slow (phase 1) nitrate consumption, then the consumption rate increased rapidly for a few days (phase 2), at which point it plateaus (phase 3), and eventually decreases and ceases (phase 4).

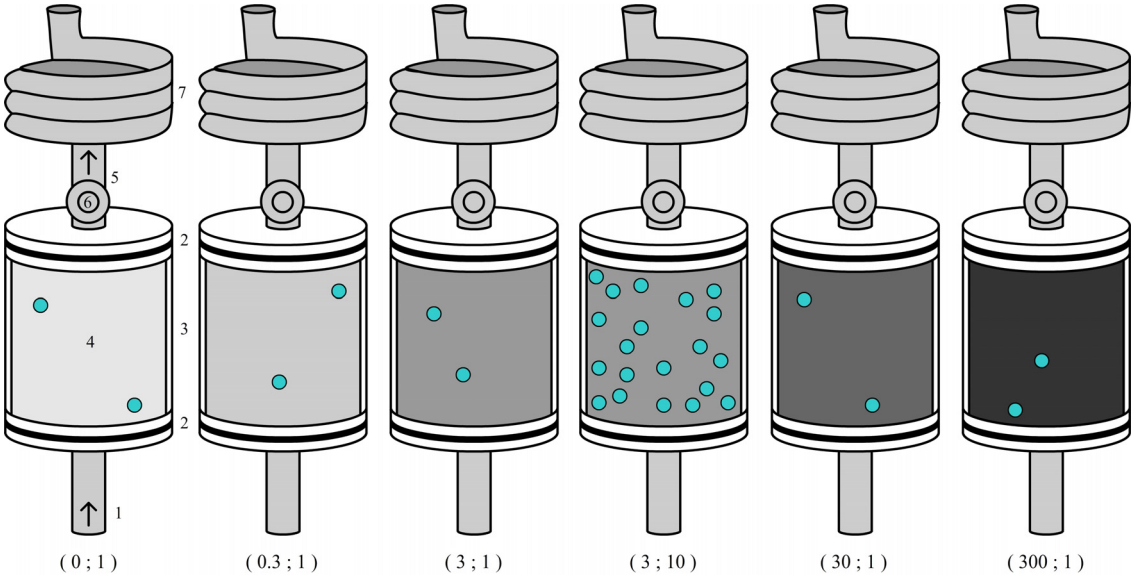

Figure 8: Array of 225 ml columns. The columns contained an increasing amount of soil (0g, 0.3g, 3.0g, 3.0, 30g, and 300g, indicated by the first number in the brackets) with the rest of the volume being occupied by sand. All were inoculated with the same amount of bacteria, previously extracted from soil, with the exception of column 4, which was inoculated with ten times as many bacteria as the other columns. Relative inoculum is indicated by the second number in the brackets, and by the abundance of dots in the column matrix. 1: stainless steel influent pipe, 2: Teflon plug with nitrile rubber gasket, 3: borosilicate glass cylinder, 4: central compartment for soil, 5: stainless steel effluent pipe, 6: port for withdrawing samples, 7: stainless steel coil for storing effluent.

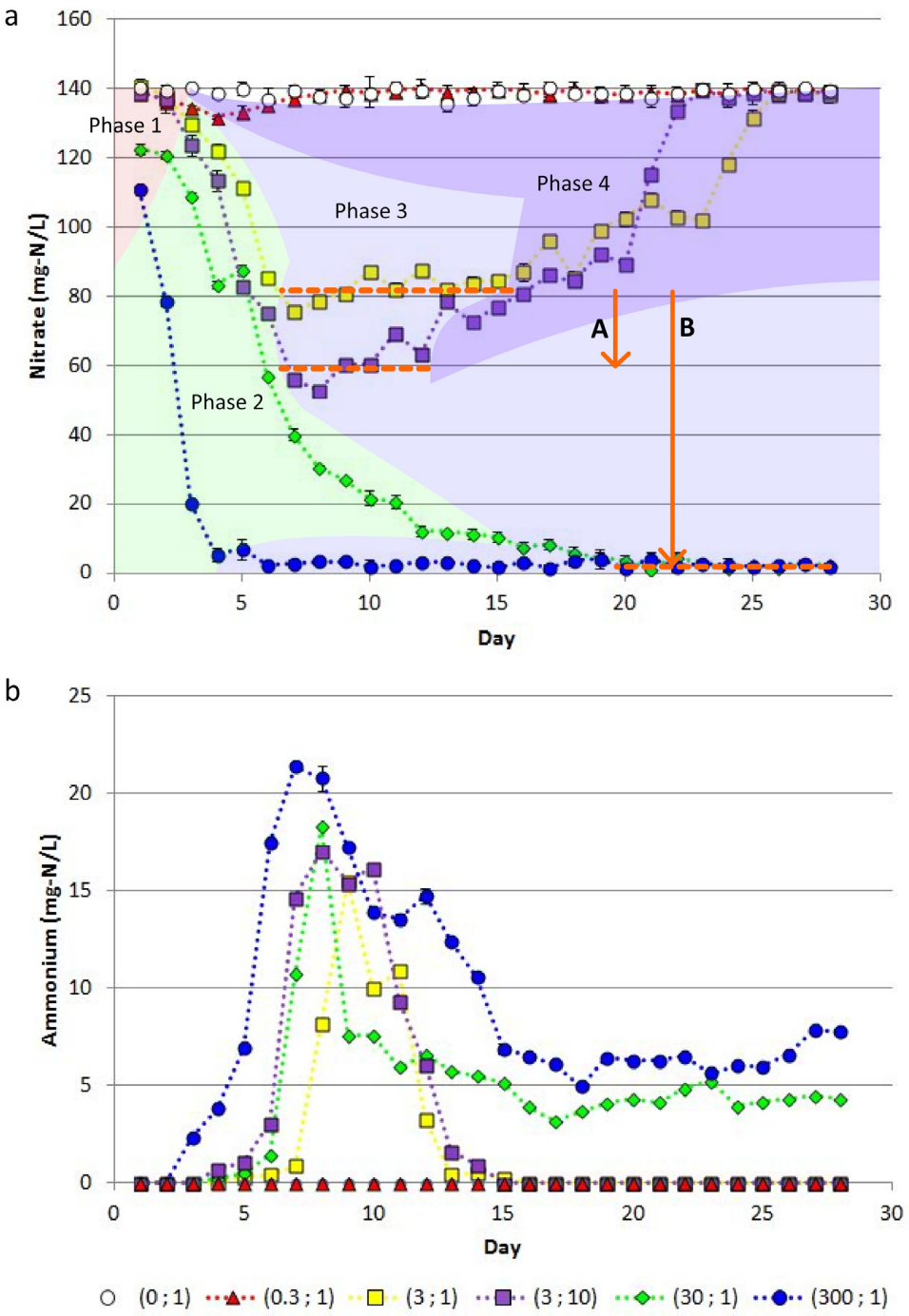

Figure 9: Column effluent concentrations of nitrate (a) and ammonium (b). Phase 1: Initial slow nitrate consumption. Phase 2: Rapid increase in nitrate consumption. Phase 3: Plateau in nitrate consumption. Phase 4: Decreasing nitrate consumption. Arrow "A" indicates the effect of increasing the inoculum tenfold, arrow "B" the effect of increasing the amount of available carbon. The first number in a bracket indicates the amount of soil in a given column, the second number indicates the size of its initial inoculum relative to the lowest overall initial inoculum. For most points the ±SE bars are smaller than the symbol size.

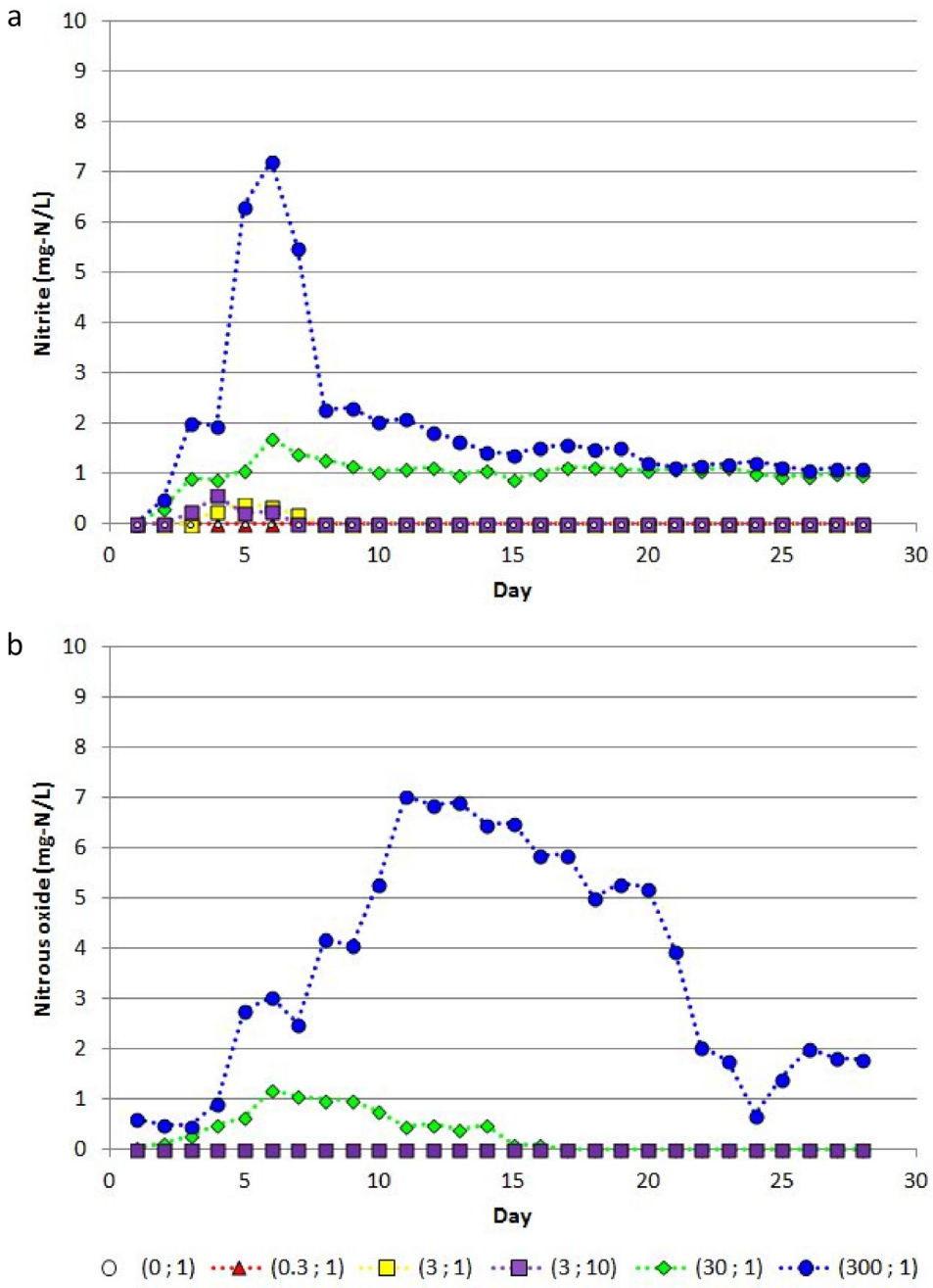

Figure 10: Column effluent concentrations of nitrite (a) and nitrous oxide (b). The first number in a bracket indicates the amount of soil in a given column, the second number indicates the size of its initial inoculum relative to the lowest overall initial inoculum. For all points the ±SE bars are smaller than the symbol size.

It is assumed that phase 1 is caused by the limited population needing to multiply before nitrate-transformations could take off, and once that had happened a progressive increase in nitrate consumption was seen (phase 2). At this point the columns are no longer limited by microbial abundance and

start becoming limited by carbon in phase 3, and nitrate consumption at this point starts being affected by the store of organic matter starting to run out throughout phase 4. The columns packed with 30 and 300g soil never got to phase 4 due to their larger electron donor supplies.

As per figures 9 and 10, production of nitrite, ammonium and nitrous oxide during nitrate-reduction in the *F. sylvatica* forest soil column microcosms was seen to follow a sequential pattern: an initial burst of nitrite production led to DNRA, and for the microcosms which became mass transfer limited with respect to nitrate also to nitrous oxide production.

In figure 11 it is seen that increasing the initial store of electron donor increased both nitrate consumption and ammonium, nitrite and nitrous oxide production. The increase was not linear, and nitrite and nitrous oxide were only produced in significant amounts in the columns which became mass transfer limited with respect to nitrate. Reducing biokinetic limitation by increasing the initial inoculum led to a minor increase in nitrate-reduction, and a very substantial 71% increase in DNRA.

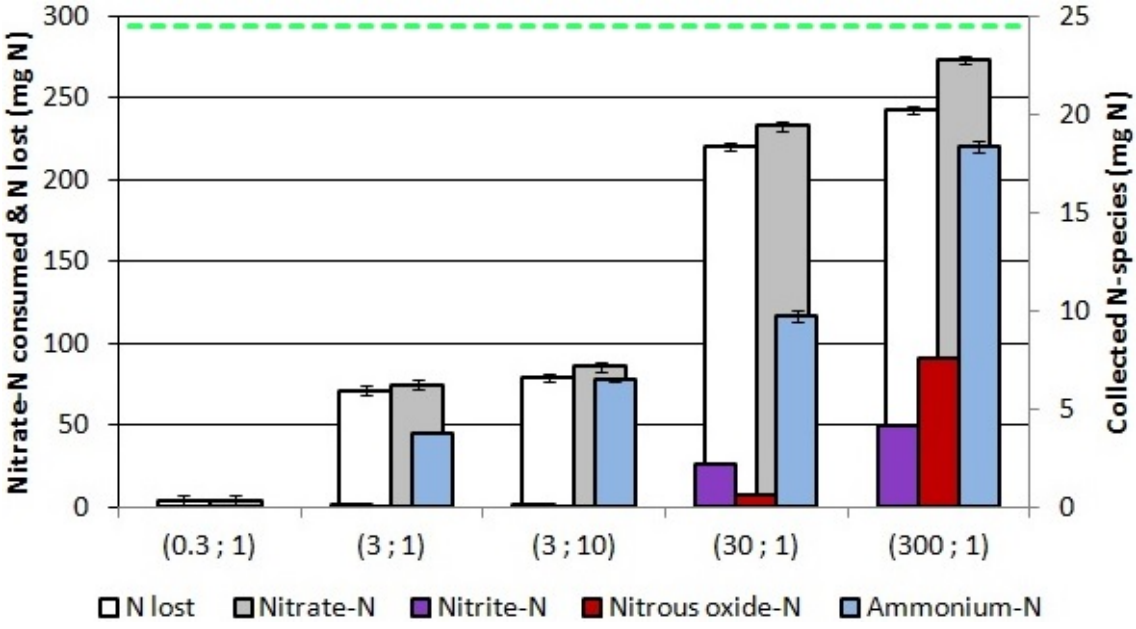

Figure 11: Nitrate consumption in columns and nitrogen compounds collected in effluent. The dashed line in (a) indicates the total amount of Nitrate-N added to each column, 294mg. The first number in a bracket indicates the amount of soil in a given column, the second number indicates the size of its initial inoculum relative to the lowest overall initial inoculum. Please note that the left and right vertical axes have different scales. For some values the ±SE bars are too small to be visible.

Additionally the mass transfer limited columns achieved complete nitrate-removal, while simultaneously maintaining both DNRA and incomplete denitrification, with nitrite and ammonium-production rates of 1 and 5 mg-N/l respectively, suggesting that DNRA is an integral part of the ecosystem.

The sequential nature of the nitrite and ammonium peaks suggests that similar mechanisms are at work in the involved columns. A sudden activity increase could have caused the initial burst of nitrite, while the following ammonium production could have resulted from easily diffusible organic compounds creating a high carbon flux early on, which would be in line with the many published studies saying that high carbon/nitrogen ratios favor DNRA (Fazzolari et al., 1998; Kraft et al., 2014; Silver et al., 2005; Smith, 1982).

Roughly 4% DNRA and 1% incomplete denitrification was seen during 100% nitrate reduction in the columns with 30 and 300g soil, which is surprising as DNRA is thought to be favored by a high electron donor/acceptor ratio, and incomplete denitrification is expected to relate to the electron donor being limiting. Either the microbial community included both DNRA microbes and microbes with incomplete denitrification, or the system contained hotspots in which DNRA was favored, and others favoring incomplete denitrification.

2.1 Protocol for experimentally imposing several quantifiable degrees of diffusive limitation on the system

In order to investigate if modulating diffusive carbon limitation would impact nitrogen cycling in the system I had to develop and implement a protocol for experimentally imposing several quantifiable degrees of diffusive limitation on the system.

Quantifying the degree of diffusive limitation could be done using the third Damköhler number presented in section 1.1.1. using:

$$Da_3 = \text{biodegradation rate / diffusion rate} = k_{bio} \cdot R^2 / D_{eff} \qquad (1)$$

, where k_{bio} is the biotransformation rate constant, R is the aggregate radius, and D_{eff} is the effective intra-aggregate diffusion coefficient that accounts for sorption-retarded diffusion within aggregates. However this would

necessitate that soil aggregates with a clearly defined interface to their surroundings could be procured.

I chose to work on achieving this goal by encasing sterile soil in an alginate matrix. Alginate has been used to encapsulate and thus immobilize bacteria in several studies looking at how the bacteria degrade pollutants (Fang et al., 2014; Hermanowicz et al., 2003), and here the alginate aggregates would mobilize soil instead of bacteria.

After testing different concentrations of soil and alginate, and different methods of casting the soil-alginate aggregates, the following protocol was developed: Sieved soil was mixed with an 1.0% solution of sodium alginate which had been sparged with nitrogen gas for 20 minutes to minimize the amount of dissolved oxygen. The mixture was transferred to syringes with different diameter nozzles, and subsequently firmed up by gently emptying the syringes into a 1.0% calcium chloride solution which had also been sparged with nitrogen gas for 20 minutes. Dripping the mixture into the solution formed round aggregates, and squeezing it out under the surface of the liquid formed cylindrical aggregates, as in figure 12.

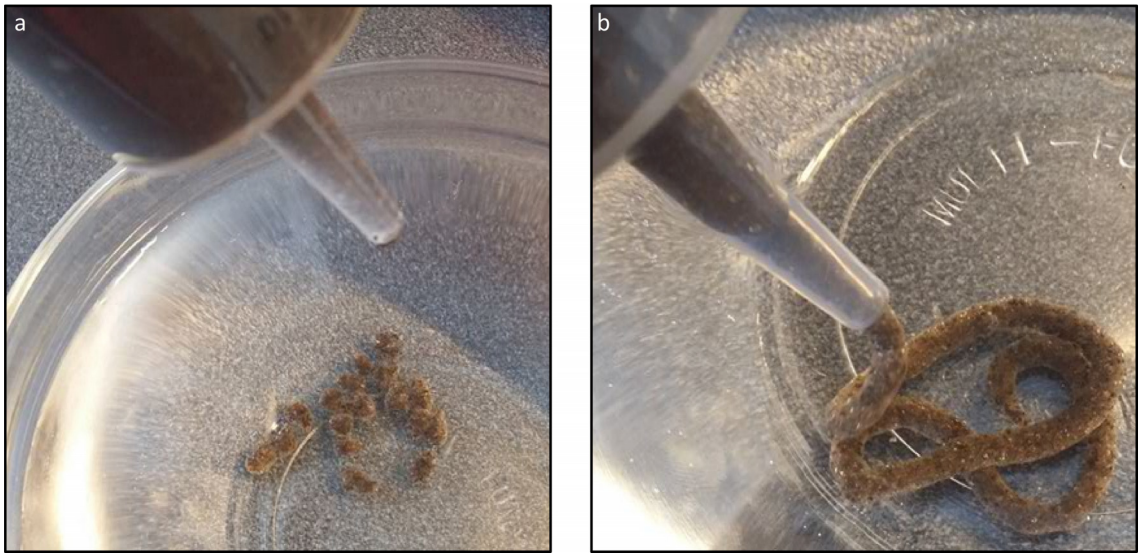

Figure 12 - Producing soil-alginate aggregates. A syringe has been filled with a mixture of soil and alginate in water, and is about to be converted into firm aggregates. In 'a' the mixture is being dripped into a calcium chloride bath to form round aggregates, and in 'b' it is being squeezed out while the syringe nozzle is under the liquid surface of the calcium chloride bath, forming a cylindrical aggregate.

Both cylinders and pellets were allowed to firm up in the calcium chloride bath for 12h hours, and were inspected visually before use; fused and otherwise non-spherical pellets were discarded. In order to have a wide range of diffusive limitation three aggregate sizes were manufactured: round aggregates with a diameter of 2mm, round aggregates with a diameter of 7 mm, and cylindrical aggregates with a diameter of 15 mm.

With the ability to produce clearly defined aggregates the organic matter composition of soil suspension from a *F. sylvatica* forest in Zealand, Denmark was taken from (Strobel et al., 2001), and the Wilke-Chang correlation was used to estimate the diffusion coefficient for the compounds in the soil suspension:

$$D_{AB} = (7{,}4 \cdot 10^{-8} (\varphi_B \cdot M_B)^{(1/2)} \cdot T) / (\eta_B \cdot V_A{}^{(0.6)}) \qquad (2)$$

, where D_{AB} is the diffusion coefficient of a compound, designated *A,* in a solvent, designated *B,* in (cm^2 / s), here D_{AB} is equal to D_W, the diffusion coefficient in water, since the solvent is water; T is the absolute temperature in K; η_B is the viscosity of the liquid medium in (kg/ (m s)) (1.308 for water); φ_B is an association parameter for the solvent (2.6 for water); M_B is the molecular weight of the liquid medium (18.02 g/mol for water) and V_A is the solute specific molar volume at its normal boiling point in (m^3 / kg mol) (Wilke and Chang, 1955), which is estimated using:

$$V_A = 0.32 \cdot L \cdot (L - 1) + \sum (A_j \cdot G_j) \qquad (3)$$

, where L is the number of C, B, and Si atoms in the longest chain, corrected for side groups, A_j is the number of side groups j, and G_j is the group contribution for group j, given by (Schotte, 1992).

To take into account that the organic substrate diffuses in a porous medium, D_w was converted to D_{eff} using:

$$D_{eff} = D_w \cdot \varepsilon^{(4/3)} \qquad (4)$$

, where ε, the porosity, is defined as cm^3 pore volume per cm^3 bed material (Millington and Quirk, 1961).

Multiplying D_{eff} for each organic compound with its concentration, summing the results and dividing it with the sum of the concentrations gave a weighted

mean D_{eff} of 7.761 cm^2/s. Together with k_{bio} which had been estimated in a batch experiment, the mean D_{eff} value was used to calculate Da_3 for the three aggregate sizes using equation (1). 21.2 ml soil-alginate aggregate was designed to contain 3.0g soil, and for the cylinder, and the 7 mm and 2 mm round aggregates that aggregate volume has a soil-water interfacial area of 60.0, 181 and 79.6 cm^2, and Da_3 values of 1.34, 0.292 and 0.0239 respectively. This corresponds to a range of diffusive limitation going from intra-aggregate diffusion limiting biotransformation, to no diffusive limitation on biotransformation.

2.2 Impact of modulating diffusive carbon limitation on nitrate reduction

A second array of column microcosms was packed with soil-alginate aggregates of varying size, inoculated with soil bacteria and fed a nitrate solution in order to investigate the impact of modulating diffusive carbon limitation on nitrogen cycling in the system. The array of columns is seen in figure 13.

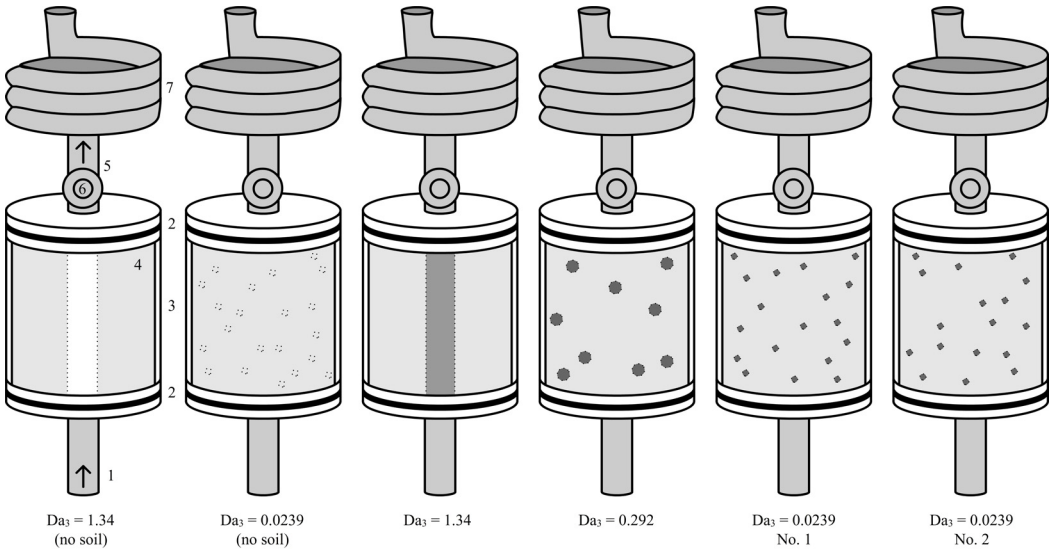

Figure 13: Array of 225 ml columns for studying the impact of diffusive limitation on the fate of nitrogen in soil. The columns were packed with similar amounts of sand and soil encased in alginate, but differing in the diameters of the soil-alginate particles, and thus also in the degree of diffusive limitation, indicated by size of the third Damköhler number, Da_3. All columns were inoculated with the same amount of bacteria previously isolated from soil. 1: stainless steel influent pipe, 2: Teflon plug with nitrile rubber gasket, 3: borosilicate glass cylinder, 4: central compartment for soil, 5: stainless steel effluent pipe, 6: port for withdrawing samples, 7: stainless steel coil for storing effluent.

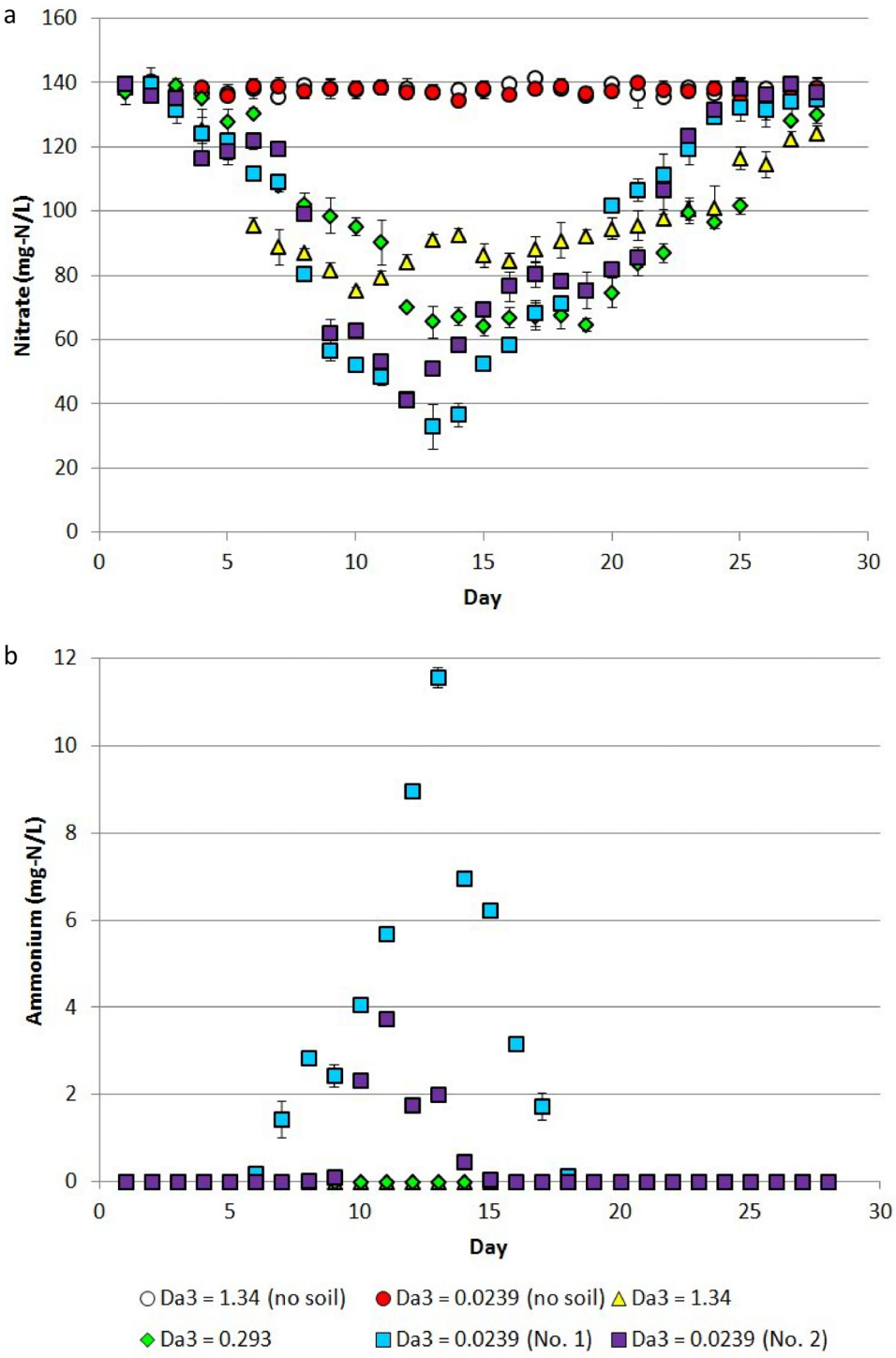

Figure 14: Effluent concentrations of nitrate (a) and ammonium (b) for different degrees of diffusive limitation quantified using the Damköhler number Da_3. For many points the ±SE bars are smaller than the symbol size.

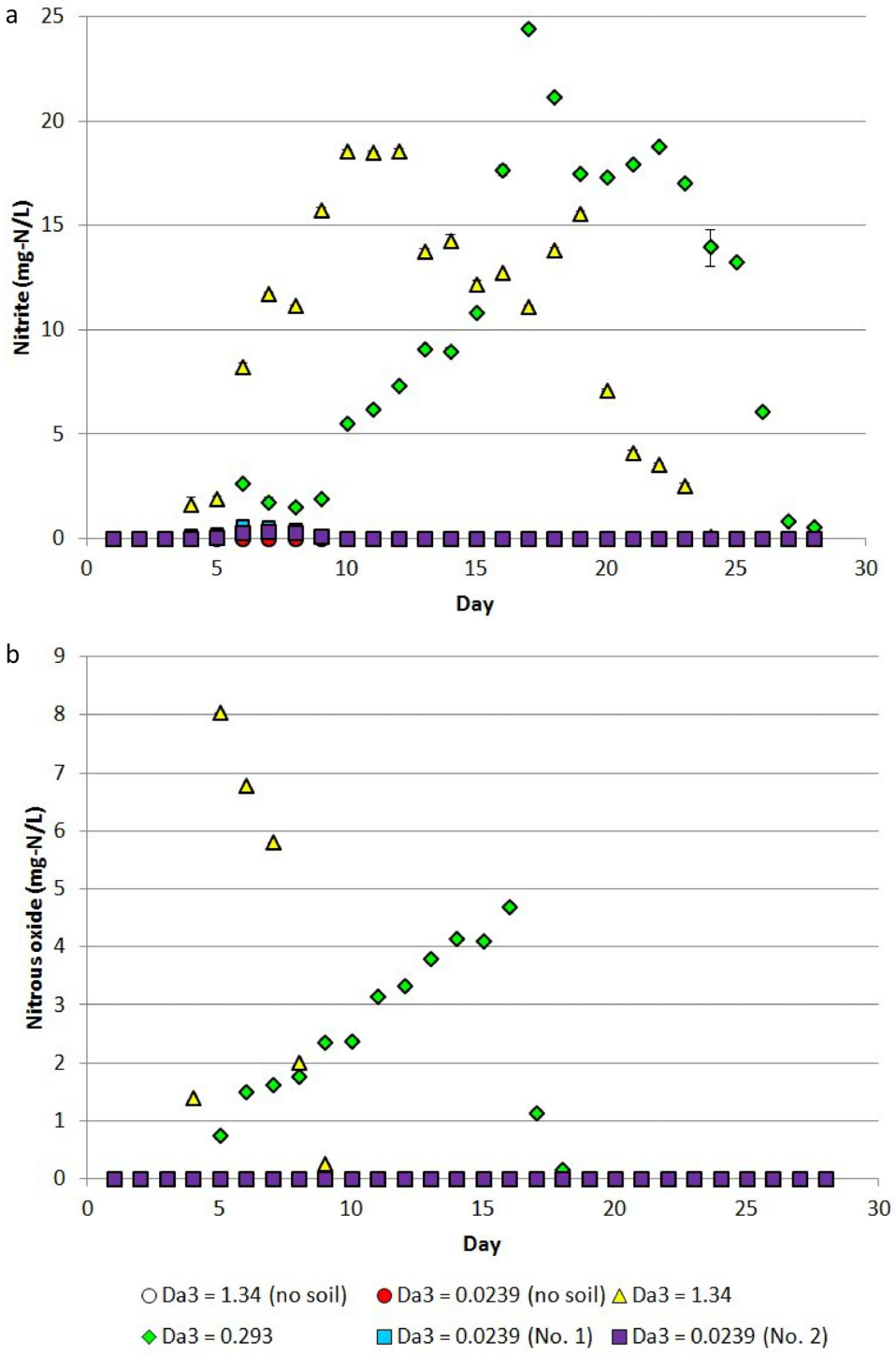

Figure 15: Effluent concentrations of nitrite (a) and nitrous oxide (b) for different degrees of diffusive limitation quantified using the Damköhler number Da_3. For many points the ±SE bars are smaller than the symbol size.

Again nitrate consumption was seen to fall in four phases. As seen in figure 14a initially it was very slow (phase 1), then increased rapidly for a few days (phase 2), after which it plateaued (phase 3), and eventually decreased and stopped (phase 4). Presumably phase 1 was caused by the limited starting population needing to multiply before nitrate transformations could increase steadily. At this point the columns were no longer limited by microbial abundance but soon hereafter carbon limitation set in at the beginning of phase 3 due to the columns starting to become limited by carbon, which eventually became so depleted that consumption started to cease in phase 4.

In the most diffusion-limited column and the one with the intermediary degree of diffusive limitation, both nitrite and nitrous oxide production began and peaked during the transition to beginning carbon limitation, and then decreased as carbon supplies dwindled further, as shown in figure 15. Under the lowest degree of diffusive limitation ammonium production began before carbon limitation had set in. It too started to cease as carbon limitation set in, and eventually died out, as shown in figure 14b.

It stands to reason that going from a high to a low degree of diffusive carbon limitation resulted in a shift from conditions that favour nitrite and nitrous oxide production, to ones favouring DNRA. This is in line with the studies that have found that plentiful availability of carbon favours DNRA over denitrification (Fazzolari et al., 1998; Kraft et al., 2014; Silver et al., 2005; Smith, 1982). Furthermore this suggests that while the bulk availability of carbon and the electron donor/acceptor ratio investigated in earlier studies may at times be a useful indicator for what nitrogen transformation processes are likely to be dominant in a given system, it is the availability and ratio locally in soil, at small scales, which decide which processes are favoured.

Looking at the literature describing the relationship between the electron donor/acceptor ratio and DNRA, a study utilizing rapidly shaken batch cultures of the soil bacteria *Citrobacter* C48 in a liquid growth media found that a high ratio led to increased DNRA (Smith, 1982), and similar results were found by a study which did bulk measurements on compacted, homogenized soil from a cultivated field (Fazzolari et al., 1998). Conversely, a study doing measurements on tropical soil cores that had been retrieved with great care in terms of preserving their internal structure found no impact of electron donor/acceptor ratio on DNRA (Sotta et al., 2008).

These studies did not look at how mass transfer limitations might have affected the electron donor and electron acceptor fluxes at the micro scale,

but it seems plausible that there were differences in the degree of diffusive limitation between the carefully preserved soil, the homogenized soil, and certainly the rapidly shaken liquid growth media . Based on the results presented here it is likely that diffusive limitation impacted the results in the aforementioned studies. Looking at the two extremes, the shaken batch cultures and the carefully preserved cores, a high electron donor/acceptor ratio would in the former be distributed evenly throughout the system, while the ratio would be impacted by extensive heterogeneity in the latter, resulting in some column areas favoring DNRA, and others favouring denitrification.

2.3 Nitrous oxide production during nitrate removal

Given that dinitrogen production is difficult to quantify, hotspots and periods of high denitrification have mostly been studied based on the denitrification intermediates nitric oxide and nitrous oxide that are of high environmental concern, the former being rapidly oxidized in air to nitrogen oxides, and the latter an important greenhouse gas, with a global warming potential per unit mass 300 times higher than carbon dioxide (USEPA, 2013).

It has been estimated that the nitrogen demand of a growing forest is between 5 and 10 kg reactive nitrogen per hectare per year, if it receives more it will lead to the system becoming enriched in reactive nitrogen (Scarascia-Mugnozza et al., 2000). This means higher availability of reactive nitrogen for plants and microorganisms, increased nitrate concentration in the soil solution (Kristensen et al., 2004; Manning et al., 2008) and increased emission of not just dinitrogen, but also nitrous oxide (Brumme and Beese, 1992; Pilegaard et al., 2006; Skiba et al., 2006). Several studies have estimated to which extent the addition of reactive nitrogen to forest ecosystems lead to increased emissions of nitrous oxide. A regression type approach with continuous nitrous oxide emission measurements found that 1.4% of the added reactive nitrogen got released as nitrous oxide in coniferous forests, and that this increased to 5.4% for deciduous forest (Butterbach-Bahl et al., 1998). This is in the same range as the 3% found for a mixed forest using a gradient approach (Skiba et al., 2006), and also in line with a recent review which found that the emission was 3.7% for coniferous forests and 5.7% for deciduous forest (Denier van der Gon and Bleeker, 2005).

There can be wide variations in yearly nitrous oxide emissions from one area to the next (DeVries et al., 2011), and there is also marked spatial and temporal heterogeneity on smaller scales. Some nitrous oxide emissions come

in the form of short pulses, lasting as little as a few days, but nevertheless contribute up to 80% of the emissions from a given ecosystems. Such emissions relate to freeze-thawing transitions (Christensen and Tiedje, 1990; Wolf et al., 2010), and also fertilization and irrigation/drainage events (Dobbie et al., 1999). Such brief but intense nitrogen cycling events underline that much nitrogen cycling is concentrated in small areas and timeframes (Groffman et al., 2009), which in return underlines the need for studying nitrogen cycling at these scales.

In the first column experiment the columns with 30 and 300g soil both saw nitrous oxide production beginning at the same time as nitrite appeared in the effluent. While it died out around day 15 for the column with 30g soil, for the one with 300g it takes off in earnest after the column becomes mass transfer limited with respect to nitrate, peaks after nitrite production peaked and reaches the same maximum rate of 7 mg-N/l as nitrite production did as shown in figure 10.

These sequential peaks suggests that part of the nitrogen that was initially turned into nitrite got directed towards nitrous oxide shortly thereafter. Nitrous oxide production from soil ecosystems is often considered in a denitrification context, even though some microorganisms which perform DNRA also produce nitrous oxide (Bleakley and Tiedje, 1982; Cole, 1988). Nitrous oxide production has been suggested to be an attempt at avoiding high concentrations of nitrite (Kaspar, 1982), this is in line with our observation of the nitrite peak being followed by nitrous oxide production.

In the second column experiment where different degrees of diffusive carbon limitation was imposed on the system no nitrous oxide production was registered during DNRA, only during denitrification. With a high degree of diffusive carbon limitation nitrite and nitrous oxide production accounted for 20% and 2.3% of the total nitrate reduction, respectively. This decreased to 0.2% and 0% under conditions of low diffusive limitation, as shown in figure 16. It has been suggested that nitrous oxide emission is related to environmental conditions, and not recognizably so to soil origin (van den Heuvel et al., 2009). This is in line with the column experiment showing that the system could be pushed from incomplete denitrification with production of nitrous oxide, to DNRA with no production of nitrous oxide, by decreasing the diffusive limitation on carbon.

While both DNRA and nitrous oxide emission was impacted by reducing the degree of diffusive carbon limitation, it should be noted that a linear

relationship between diffusion limitation and nitrous oxide emission was not observed. As shown in figure 16 going from a high degree of diffusive limitation to an intermediary one increased the release of nitrous oxide slightly, and further reduction of diffusive limitation was needed to switch it off. This shows that while increasing diffusive carbon limitation can reduce nitrous oxide emission, further research is required to elucidate the finer details in the relationship between diffusive limitation and nitrous oxide emission.

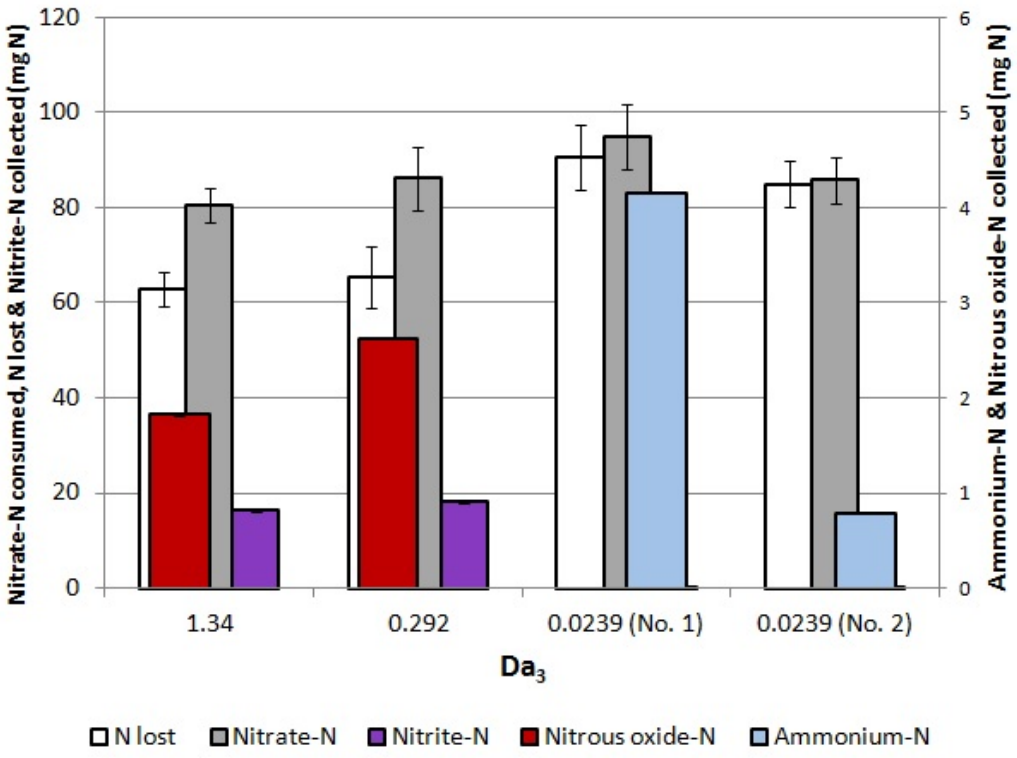

Figure 16: Nitrate consumption in columns (a) and nitrogen compounds collected in effluent (b) as a function of diffusive limitation quantified using the Damköhler number Da_3. Please note that the left and right vertical axes have different scales. For some values the ±SE bars are too small to be visible.

3 Studying soil and sediment heterogeneiety at the micro scale

The use of non-microscale tools for measuring microscale features risks overlooking them or leaving them represented by a single data point (Docekalová et al., 2002; Grundmanis and Murray, 1977; Leermakers et al., 2005), and the use of microscale tools has been shown to be required for accurately describing microscale gradients, exemplified by microscale studies of the diffusive boundary layer in the western South Atlantic (Wenzhöfer et al., 2001) and steep nitrate gradients in sediment dominated by iron and sulphate reduction zones (Mortimer et al., 2002). Kuzyakov and Blagodatskaya have recently reviewed the size, occurence, spatial distribution, lifetime and associated intensity of microbial activity of environmental microbial hotspots. Their work linked the hotspots, their growth, competition and consumption of labile carbon, ultimately concluding that despite occupying less than 5% of the soil volume, the hotspots may be responsible for most processes measured in mixed soil samples (Kuzyakov and Blagodatskaya, 2015). The growing realization that nitrogen cycling and other important soil processes are concentrated in small areas and timeframes not merely mandate further studies into these processes, it also underlines the need for tools capable of studying them on such scales, and for these tools to become increasingly accessible to a wide selection of the scientific community. Some tools for studying soil and sediment heterogeneity at the micro scale such as microelectrodes and microoptodes are very well established in environmental research, while others are either relatively new developments and/or currently underutilized due to cost or the need for specialized training to manufacture and/or use them.

There exists an extended body of work on microelectrodes and microoptodes part of which has already been reviewed. One review gives fairly high prominence to a number of commercially available ion selective electrodes targeted at analytes such as halide and heavy metal ions, without including microsensors for pH, O_2, N_2O, H_2, H_2S, CO_2 and redox (Hanrahan et al., 2004), all of them environmentally interesting analytes. Another includes microelectrodes for most of these analytes, but pays little attention to consistently showing key sensor performance characteristics such as resolution, response time and interferences (Taillefert et al., 2000). This weakness is shared by a third review (Viollier et al., 2003), which nevertheless complements the section on electrochemical sensors with

sections on optical microsensors and planar gel sensors, albeit with the same low attention to performance characteristics as for electrochemical sensors. Then there are a number of quite admirable reviews on individual microsensor categories, ranging from potentiometric microsensors (de Beer, 2000), microoptodes (Kühl, 2005), oxygen microsensors (Glud et al., 2000), sulphide microsensors (Kühl and Steuckart, 2000) and microbiosensors (Revsbech et al., 2000). These reviews give excellent introductions to their respective microsensors, without in themselves supplying the broader perspective of microscale tools available to environmental studies.

While planar sensors are a very interesting development for environmental microscale studies, they have enjoyed little attention from reviewers. A recent review briefly mentions planar oxygen optodes while reviewing tools for studying spatial and temporal oxygen dynamics in sediments (Satoh and Okabe, 2013) and another did this in greater detail five years earlier (Glud, 2008), but the focus of the review limited it to planar O_2 optodes. The hitherto most comprehensive review including planar optodes focuses on pH and O_2 measurements in sediment using microsensors and planar optodes, but also includes CO_2 planar optodes and planar gel probes (Stockdale et al., 2009). While this review gives a comprehensive introduction to tools for microscale studies in sediments, a fair amount of the sensors included in the review are not nearly the sensors with the highest possible resolution in their group at the time the review was published, and for some calling their resolution 'microscale' is being somewhat generous. The review does cover some key performance characteristics, but gives precious little information on important details such as potential interferences and sensor lifetimes.

I reviewed a broad selection of both microsensors and planar sensors for use in environmental studies, providing the reader with a broad perspective with clear presentations of key performance characteristics for individual sensors, and complemented this with suggestions for techniques for providing spatial data on the location of microorganisms and the structures they inhabit. For the purpose of keeping the paper from becoming too wide in scope it reserves the term 'microscale' for structures, tools and processes measuring or occurring at scales from 1 to 100 µm, excluding 'macro-' and 'mesoscale' tools which it defines as being from 1 cm and above and in between 'micro-' and 'macroscale', respectively.

The core outcome of the reviewing efforts follow below.

3.1 Microsensors

Microsensors cover both microelectrodes, microoptodes and microbiosensors, and each of these groups include several individual sensor types. Most of them are amongst the most accessible tools for microscale measurements in environmental systems, and are basically pen-shaped probes with tips measuring as little as a micrometer, capable of converting a non-electrical physical or chemical input into an electrical or optical output. The measurements are performed at the scale of the tip, giving the microsensors high spatial resolution, and their minute internal dimensions give short diffusive distances and consequently fast response times (Glud et al., 2000; Kühl and Revsbech, 2001; Revsbech and Jørgensen, 1986).

As microsensors have been an established technology in environmental research for several decades there exists an extended body of work. Broadly speaking, microsensors fall into two categories: microelectrodes based on electrochemical signal detection, and microoptodes based on optical signal detection. Both simple solid state, gas and ion exchange microelectrodes have been manufactured with tips, and thus resolutions, at, or below, 10 µm. The fastest have response times around 1 s, and most are at, or below, 10s. The published lifetimes generally favor the gas sensors, but many will function for about one month, see table 1 for an overview.

While many of the first microelectrodes relied on electrochemical sensing principles, there are environmental variables that cannot be measured using this approach. In some cases this problem can be overcome by combining traditional electrochemical microsensors with biological catalysts. This is complicated by the fact that the biological catalysts generally need specific physicochemical conditions to be able to function, and there is a limit to how long these conditions can be maintained inside the microsensor due to consumption of nutrients and production of metabolites. Tip diameters are around 20 µm, two times or more than that of a microelectrode, response times are often close to 60 seconds, and the sensors have lifespans in the range of a few days, see table 2 for an overview.

Microoptodes have been manufactured with low response times (~1 s), good long-term signal stability and, unlike most non-optical microsensors, they are not affected by high concentrations of H_2S. Depending on the sensor chemistry they may be vulnerable to high concentrations of some compounds such as SO_2, see table 1, and also to high pressure(Glud et al., 1999).

Table 1 - A selection of microsensors and their performance characteristics.

Sensor type	Minimum tip diameter (µm)	Maximum response time to 90% signal (s)	Major interferences	Minimum expected lifetime (months)	References
Gas sensors					
O_2	1	0.5	H_2S	12	(Glud et al., 2000)
H_2S	2	0.5	light	12	(Kühl and Steuckart, 2000; Kühl et al., 1998)
H_2	1	10	H_2S	12	(Ebert and Brune, 1997; Witty, 1991)
N_2O	1	50	H_2S	12	(Andersen et al., 2001)
CO_2	10	300	H_2S	days	(Cai and Reimers, 1993)
Ion exchange sensors					
pH	10	10	none	12	(Amman, 1986; Revsbech and Jørgensen, 1986; Thomas, 1978)
NO_2^-	10	15	H_2S, Cl^-	<1	(de Beer, 2000; deBeer et al., 1997b)
NO_3^-	1	30	H_2S, Cl^-, HCO_3^-	hours	(de Beer and Sweerts, 1989; de Beer, 2000)
CO_3^-	1	5	none	days	(Choi et al., 2002)
NH_4^+	1	60	Na^+, K^+	days	(de Beer and van den Heuvel, 1988; de Beer, 2000)
Ca^{2+}	1	5	none	days	(Amman, 1986; de Beer, 2000)
pH	1	10	none	<1	(de Beer, 2000; deBeer et al., 1997a; Lee and de Beer, 1995)
CO_2	10	10	none	<1	(de Beer, 2000; deBeer et al., 1997a; Lee and de Beer, 1995)
Optodes					
O_2	20	1	SO_2, background fluorescence	<1	(Glud et al., 2000; Klimant et al., 1997b, 1995)
Temperature	20	not published	O_2	1	(Holst et al., 1997)
pH	20	60	none	1	(Kohls et al., 1997)
Solid state sensors					
pH	10	60	H_2S	<1	(Vanhoudt et al., 1992)
Redox	10	not published	H_2S	12	(Ebert and Brune, 1997)
S^{2-}	10	900	pH	days	(Kuhl and Jørgensen, 1992; Revsbech and Jørgensen, 1986; Visscher et al., 1991)

3.2 Planar sensors

Compared to microsensors, planar sensors capable of performing microscale measurements in environmental settings are a relatively new development. They include planar gel probes and planar optodes.

The gel probes are divided in those that rely on *d*iffusive *e*quilibration between a *t*hin-film of a gel (DET) and the pore water (Davison et al., 1991), and those that rely on the establishment of a *d*iffusive *g*radient in a *t*hin-film of gel (DGT) (Davison et al., 1994). DET has been used for Fe, Mn (Davison et al., 1994), alkalinity, Br, Cl, Ca, CO_2, NH_4^+ (Mortimer et al., 1998), NO_3^-, SO_4^{2-} (Krom et al., 1994), K, Mg (Zhang et al., 1999), Cd, Cr, Cu, Pb, Zn (Yu et al., 2000), Mo, Re, and U measurements (Morford et al., 2003); DGT has been used for As, Mn, Zn (Davison et al., 1997), Co, Fe, Ni, and S^{2-} (Motelica-Heino et al., 2003), Cd, Cu (Buzier et al., 2006), and Pb measurements (van der Veeken et al., 2008).

Table 2 - A selection of microbiosensors and their performance characteristics.

Sensor type	Minimum tip diameter (µm)	Maximum response time to 90% signal (s)	Major interferences	Minimum expected lifetime (months)	References
NO_2^-	20	180	H_2S	days	(Larsen et al., 1997; Nielsen et al., 2004)
NO_x^-	20	60	H_2S	days	(Larsen et al., 1997; Revsbech et al., 2000)
CH_4	20	60	H_2S	<1	(Damgaard and Revsbech, 1997; Damgaard et al., 1998; Revsbech et al., 2000)
Glucose	10	10	H_2S	days	(Cronenberg et al., 1991)
Volatile fatty acids	20	90	H_2S	days	(Meyer et al., 2002)
Dissolved organic carbon	50	10	O_2	days	(Neudörfer and Meyer-Reil, 1997)

Planar optodes are a newer development than planar gel probes, and have a sensing principle similar to that of microoptodes, but here the sensing membrane in the tip of the microoptode has been scaled up significantly, and the signal is recorded using a camera and analyzed using a computer (Glud, 1998; Glud et al., 1996).

The gel probes can be used to measure a wide range of analytes, however they do not allow for repeated measurements. Planar optodes have been developed for O_2 (König et al., 2005; Larsen et al., 2011; Oguri et al., 2006), NH_4^+ (Delin and Strömberg, 2011; Strömberg and Hulth, 2001), CO_2 (Zhu and Aller, 2010), temperature (Borisov and Klimant, 2008) and pH (Blossfeld and Gansert, 2007; Larsen et al., 2011), and multi-analyte planar optodes have also been presented (Borisov et al., 2011). Unlike the gel probes planar optodes not only allows for good spatial resolution, but also repeated measurements with high temporal resolution. The resolution of the images recorded with a planar optode depends largely on the camera and the geometry of the experimental setup. Setups with a maximum theoretical pixel resolution in the vicinity of 10 x 10 µm have been published (Larsen et al., 2011), at or near the resolution that many microelectrodes can offer in one dimension. Other performance characteristics of planar optodes also rival those of most microelectrodes: response times in the range of 10-20s are common, see table 3 for details.

3.3 Other tools

Microsensors and planar sensors can describe chemical gradients but not give information about the spatial location of microorganisms or the structures they inhabit. For this reason I direct the reader towards techniques that have been used successfully to gain such information, such as computer aided X-ray tomography, CT, and nano secondary ion mass spectrometry, NanoSIMS.

CT can be used to provide spatial data from microenvironments with negligible effect on both soil and microorganisms (Schmidt et al., 2015), and NanoSims has been used to investigate soil microparticles (Blair et al., 2006; Mueller et al., 2012), microorganisms (Keiluweit et al., 2012; Musat et al., 2008; Pumphrey et al., 2009) and interactions between organic matter and minerals (Keiluweit et al., 2012).

Table 3 – A selection of planar optodes and their performance characteristics, where available. A dash (-) indicates that information is not available. * 20s with a silicone layer to reduce light scattering, 3s without. **Response time when pH was increased from pH 6.5 to 8.9.

Sensor type	Maximum response time to 90% signal (s)	Major interferences	Minimum expected lifetime	References
Single analyte planar optodes				
O_2	-	-	> 1 month	(König et al., 2005)
O_2	10	-	> days	(Oguri et al., 2006)
O_2	3/20*	-	-	(Larsen et al., 2011)
NH_4^+	120	K^+, pH	8 months	(Strömberg and Hulth, 2001)
CO_2	18	-	2 weeks	(Zhu and Aller, 2010)
Temperature	-	O_2	<day if exposed to oxygen	(Borisov and Klimant, 2008)
pH	-	-	8 weeks	(Blossfeld and Gansert, 2007)
pH	60	-	-	(Larsen et al., 2011)
Dual analyte planar optode				
pH	-	-	> days	(Blossfeld et al., 2013)
CO_2	-	-	> days	
Dual analyte planar optode				
O_2	12	Temperature changes	-	(Borisov et al., 2011)
CO_2	122	Temperature changes	-	
Quadruple analyte planar optode				
O_2	22	Temperature changes	-	(Borisov et al., 2011)
CO_2	274	Temperature changes	-	
pH	34	Temperature changes, ionic strength	-	
Temperature	-	O_2, pH	-	

Both techniques have remarkable microscale resolutions, < 1µm for CT (Helliwell et al., 2013; Van den Bulcke et al., 2009) and as low as 50 nm for NanoSIMS (Keiluweit et al., 2012), making them potentially very valuable to future environmental microscale research.

3.4 Current state and future direction of microscale environmental studies

Advances in microscale technology have put remarkable tools at our disposal, and putting these tools to use in explaining microscale heterogeneity has filled many gaps in our knowledge, such as explaining the absence of anaerobic bacteria in anoxic niches in planktonic aggregates was due to the niches being too transient for the bacteria to become established there (Ploug et al., 1997).

Microelectrodes and gel probes exist for an impressive array of analytes, while planar optodes combines two-dimensional microscale resolution and the potential for repeated measurements over long periods of time with a less expansive array of potential analytes. Expanding this list to include compounds such as nitrate, iron and sulphide would be of great interest to ecological studies. Further efforts toward more potential analytes, higher robustness, higher accessibility, and, in the case of multi-analyte planar optodes, lower response times are needed for them to reach their full potential.

4 A nitrate sensitive planar optode

Small areas and brief periods of activity often account for a large fraction of the total nitrogen turnover, and consequently describing nitrogen cycling on the microscale is a big challenge. While some of the factors influencing nitrogen transformations are well known, the knowledge of how the factors interact at the micro scale remains low (Groffman et al., 2009), and it is not just more studies that is needed, we also need to increase our arsenal of tools for studying nitrogen cycling at small scales:

Microsensors with good performance characteristics have been produced for important reactive nitrogen species such as NO_2^-(de Beer, 2000; deBeer et al., 1997; Larsen et al., 1997), NO_3^- (de Beer and Sweerts, 1989; de Beer, 2000), N_2O (Andersen et al., 2001) and NH_4^+ (de Beer and van den Heuvel, 1988; de Beer, 2000), it also reveals a gap for planar optodes: NH_4^+ is the only reactive nitrogen species for which a planar optode has been developed (Delin and Strömberg, 2011; Strömberg and Hulth, 2001). Here I introduce a NO_3^--sensitive planar optode.

The NO_3^--sensitive planar optode is based on a pH-sensitive planar optode utilizing 1-hydroxypyrene-tris-3,6,8-octadecylsulfonamide, HPTS-TOA, in a poly vinyl chloride, PVC, matrix (Larsen et al., 2011), and utilizes a mixture of HPTS-TOA and tridodecylmethylammonium, TDMA, chloride in a PVC matrix, which enables the measurement of NO_3^- as a function of the fluorescence of the pH indicator HPTS-TOA due to coextraction mediated by TDMA (Mohr et al., 1995). Since both HPTS-TOA and TDMA have similar degrees of hydrophobicy both can be retained by the PVC matrix.

The resulting NO_3^--sensitive planar optode exhibits a linear response to nitrate from 1 to 50 mM at pH 8.0, a fast response time to 90% signal of < 10s. Comparing its performance characteristics to the planar optodes reviewed in paper III it performs very well:

- the < 10s response time is comparable with the best of them, and significantly better than the 120s reported for the NH_4^+-planar optode.
- its expected lifetime of more than two months likewise places it amongst the frontrunners, making it possible to prepare the planar optode sheets well in advance of the experiment, and still perform measurements over an extended period of time, something that is currently unfeasible for one of the published O_2 planar optodes and the europium(III)-based temperature planar optode which can have a lifetime of less than a day (Borisov and Klimant, 2008).

- it does have interferences, notably pH and chloride, but comparing this to interferences for other planar optodes is difficult due to their potential interferences being underreported. The NH_4^+-planar optode is also based on coextraction and also suffer from interference from pH, in addition to K^+ (Delin and Strömberg, 2011; Strömberg and Hulth, 2001).

The combination of the above planar optode performance characteristics and the possibility of generating data on spatial NO_3^--distributions with good temporal resolution with a consumer camera such as the Canon EOS1100D used for testing the NO_3^--planar optode makes it an interesting choice for studying nitrogen dynamics on the micro scale without the need for a large number of samples (figure 17). And as shown in paper IV its use can be combined with more conventional analytical procedures, as long as these are scaled down to smaller volumes to limit the impact of destructive extraction.

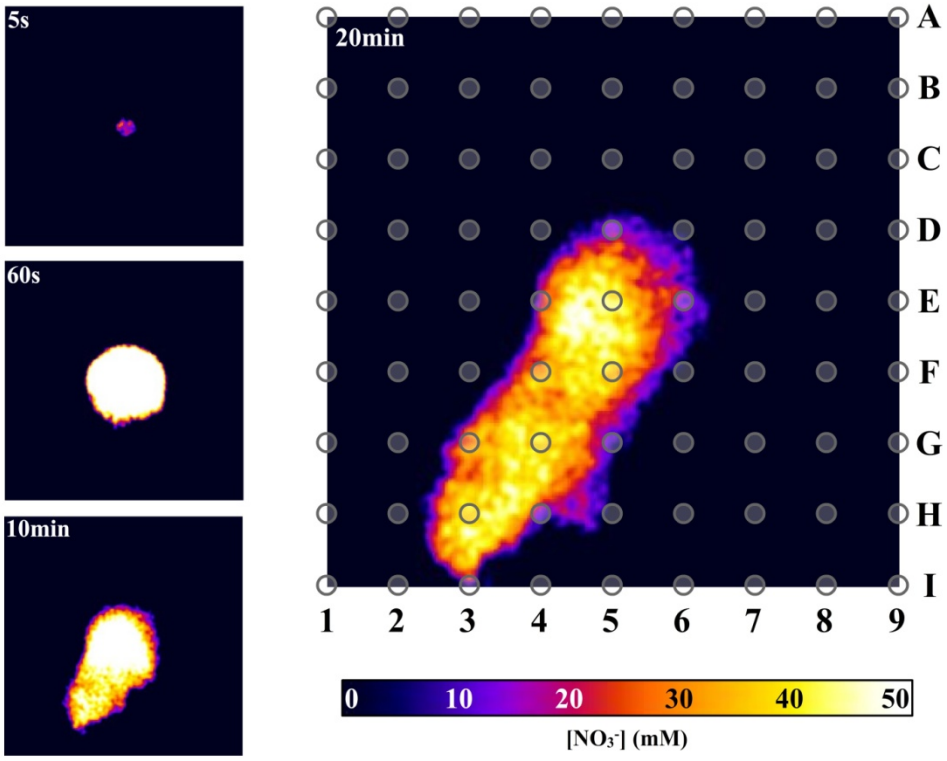

Figure 17: Planar optode images of a nitrate plume over time. 5.0 ml 50mM sodium nitrate was injected over 20 seconds in injection port E5 at t = 0. a: t = 5s. b: t = 60s. c: t = 10 min. d: t = 20 min. The injection port grid used for withdrawal of samples for classical analytical procedures is indicated by grey circles. Adapted from paper IV.

Early in the process of developing the large, high resolution nitrate sensitive planar optode the need for producing large (20x20cm) sheets of optode sensor foil arose. Literature review (paper III) and communications with other researchers revealed that people relied on spraying a mixture containing dissolved sensor chemical, matrix and a solvent onto the support foil using a paint spray gun, or utilizing knife coating, a process in which the mixture is spread onto the support foil much like one would spread butter on a slice of bread (Larsen et al., 2011). Spray coating worked well when the sensor matrix compound was silicone based, and also when no sensor matrix compound was added to the mixture being spray coated onto the support foil, but in the latter case the sensing layer exhibited poor coverage and adherence. Using polystyrene or poly vinyl chloride as matrix compound immediately resulted in formation of thin filaments which fast lead to the nozzle of the paint spray gun becoming clogged, making spray coating unfeasible for sensor layers containing these compounds. Knife coating worked acceptably for sensor sizes measuring a few cm in width and height, but when the technique was applied to the production of larger sensors results were unsatisfactory: a smooth sensing layer was not achieved for the entire sensor foil (figure 18), and when attempting to overcome this by increasing the amount of sensing mixture applied to the support foil a significant amount of the applied sensing mixture was wasted without resulting in a smooth layer.

Figure 18: 3x3 cm section of 20x20cm planar optode sensor foil produced using knife coating. Inspection by the naked eye reveals that the thickness if the sensing layer is markedly uneven.

These difficulties led to the construction of the prototype of a tool for the production of planar optode sensor sheets described in paper V. The tool has been used to successfully produce 20x20cm large sheets of planar optode sensor foil for pH and oxygen measurements, and subsequently it was used to make the sensor sheets for the nitrate sensitive planar optode described in paper IV.

Patent searches in the Derwent Innovations Index, FreePatentsOnline, Espacenet, Google Patents and the United States Patent and Trademark Office databases did not yield any patents describing similar tools for producing planar optode sensor foils, so this tool may fill a gap for researchers desiring to manufacture large planar optode sensor sheets for use in experiments. In combination with a recent work describing the construction and use of a relatively inexpensive but high resolution planar optode setup (Larsen et al., 2011), the nitrate sensitive planar optode and this tool makes it possible to successfully, and with minimal practice, start performing planar optode experiments for a range of analytes without having to make too hefty investments.

5 Conclusions

The three parts of the PhD study have made the following contributions to the study of spatial heterogeneity and nitrate metabolism in soil:

First part (Papers I & II)

- I established that both DNRA and denitrification can occur in *F. sylvatica* soil from Dyrehaven, Northern Zealand, Denmark.
- DNRA was seen to increase with increasing availability of electron donor in the form of carbon.
- At complete nitrate reduction there was overall 7% DNRA and 4% partial denitrification, suggesting that DNRA is an integral part of the ecosystem.
- A framework for quantifying diffusive limitation using the third Damköhler number and imposing it on a system was created.
- A protocol for encasing sterile soil in alginate was developed as part of the framework and used to impose several quantifiable degrees of diffusive limitation on a system.
- Going from a quantified high degree of diffusive limitation to a low was seen to shift the system from incomplete denitrification with production of nitrite and nitrous oxide, to DNRA.
- That the framework was used successfully to effect a switch in microbial metabolism when going from one quantified degree of diffusive limitation to another underlines the importance of looking at mass transfer processes quantitatively when studying processes associated with microbial hotspots.

Second part (Paper III)

A comprehensive resource describing available microscale techniques for environmental studies has been made which provides key performance characteristics and information on accessibility and system disruption for

- a broad range of available microsensors
- planar sensors, including DET and DGT probes and planar optodes, including multi-analyte planar optodes.

Furthermore the review reveals that further efforts toward higher accessibility, more potential analytes, and, in the case of multi-analyte planar optodes, lower response times are needed for the microscale tools to reach their full potential.

Third part (Papers IV & V)

- A nitrate sensitive planar optode was developed which enables the study of nitrogen dynamics on the micro scale with good temporal resolution without the need for a large number of samples.
- A tool for producing large (20x20cm) sheets of optode sensor foil was developed and used successfully to make sensor foil targeted at oxygen, pH and nitrate.
- Together the nitrate sensitive planar optode and the tool for making sensor foil makes it possible to successfully, and with minimal practice, start performing planar optode experiments for a range of analytes without having to make too hefty investments.

While the developed framework was used successfully to effect a switch in microbial metabolism when going from one quantified degree of diffusive limitation to another, something that warrants its use in the study of other microbial processes, the observation that there wasn't a linear relationship between diffusive carbon limitation and microbial metabolism shows that further research is required to shed more light on the impact of diffusive limitation on microbial nitrate reduction.

That the diffusive limitation on the carbon supply, and not the amount of carbon in the system, was shown to affect whether nitrate reduction included DNRA or incomplete denitrification, shows that the many studies published on this topic utilizing homogeneous samples and bulk measurements should be complemented with ones that take mass transfer processes into consideration.

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7 Papers

I **Pedersen, L. L.**, Dechesne, A., Jensen, M. M., Smets, B. F. (2015) Dissimilatory Nitrate Reduction to Ammonium in *Fagus sylvatica* forest soil. *Manuscript in preparation.*

II **Pedersen, L. L.**, Dechesne, A., Jensen, M. M., Smets, B. F. (2015) Reducing diffusion limitation shifts nitrate reduction metabolism from incomplete denitrification to reduction to ammonium. *Manuscript in preparation.*

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V **Pedersen, L. L.**, Smets, B. F., Dechesne, A. (2015) Notification of Invention at DTU: Planar optode sensor sheet production kit.

In this online version of the thesis, the papers are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
Water Resources Engineering, Urban Water Engineering,
Residual Resource Engineering and Environmental Chemistry & Microbiology.

The department dates back to 1865, when Ludvig August Colding, the founder of the department, gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

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